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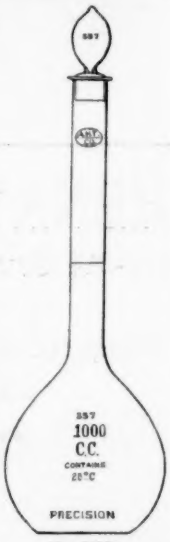

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FEEDING EXPERIMENTS WITH RAW AND BOILED CARROTS.*

By MINNA C. DENTON and EMMA KOHMAN.

(*From the Hull Physiological Laboratory, University of Chicago, Chicago.*)

(Received for publication, September 7, 1918.)

In the present food crisis, it will perhaps be conceded that our cheapest sources of calories and protein are the vegetable fats and cereal grains. It will also be readily conceded that these foods

CORRECTION.

On page 76, Vol. XXXVI, No. 1, October, 1918, under column 3 of the formula, for *d-epichitosaminic* read *d-epichondrosaminic;* under column 4 for *d-chitosaminic* read *d-chondrosaminic*.

calorie and protein values of these cooked preparations will more nearly approach those of our staple carbohydrate foods such as cereal porridges and breads, or cooked potatoes. The "watery vegetables" can then no longer be neglected as a source of fuel or calories.

As for protein, they may be so selected as to yield large amounts of nitrogen if they can be eaten in fairly concentrated form. For it will be noted that when protein (or rather, nitrogenous) calories

* We take pleasure in acknowledging our indebtedness to Major A. J. Carlson, under whose direction this work was begun, to Dr. Arno B. Luckhardt, under whom it has been continued, also to Dr. F. C. Koch, of the Department of Physiological Chemistry, who has shown us many favors in the way of laboratory facilities.

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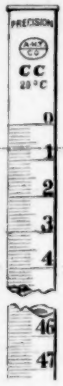

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In the present food crisis, it will perhaps be conceded that our cheapest sources of calories and protein are the vegetable fats and cereal grains. It will also be readily conceded that these foods require to be judiciously fortified or supplemented in several respects. It has often been urged that some of the common root vegetables should be used as extensively as may be practicable, as partial substitutes for the cereals, as supplements to them in certain respects, and certainly as supplements to the vegetable oils. A knowledge of the dietetic properties of these root vegetables is then of importance, particularly so since the use of dehydrated vegetables promises to come into general vogue.

In the preparation of these dehydrated products for table use it will not necessarily be the case that the excess of water contained by the vegetable in its natural state, is restored. Thus the calorie and protein values of these cooked preparations will more nearly approach those of our staple carbohydrate foods such as cereal porridges and breads, or cooked potatoes. The "watery vegetables" can then no longer be neglected as a source of fuel or calories.

As for protein, they may be so selected as to yield large amounts of nitrogen if they can be eaten in fairly concentrated form. For it will be noted that when protein (or rather, nitrogenous) calories

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are compared with total calories, spinach furnishes as large a proportion of nitrogen calories as some cuts of meat; tomatoes and cabbage more than wheat; and carrots almost as high a proportion as does corn-meal (1). Quantitatively, then, the vegetables above mentioned, when in dry form, would present equivalents or substitutes for meat, wheat, and corn-meal respectively, whether considered as fuel or as nitrogenous foods. However, it is true that the question of digestibility remains to be considered, since we live not by what we eat but by what we digest; and the quality of these vegetable proteins is all important, since in the last analysis it is not proteins as a class, but various individual amino-acids, which are able to bring about growth or to maintain nitrogenous equilibrium.

With these considerations in mind, we have instituted feeding experiments with carrots, using albino rats. They take this food readily, whether it is given raw, cooked, or dried. They become accustomed to it in a few days and increase their consumption gradually until they are in many cases taking each day an amount which (calculated as weight of fresh vegetables) is equal to or greater than their own body weight.

Three litters of rats were given equal representation in each of Lots I, II, and III, in the hope of eliminating as far as possible, the influence of individual variations.

Growth curves of Lots I, II, and III (Chart I, February 5 to March 12), show that after becoming adjusted to the food, young rats may maintain body weight without loss over a period of at least 5 weeks, on a diet of chopped carrots fortified only with suitable amounts of sodium chloride, calcium phosphate, and calcium lactate. (Some animals survive much longer than this and seem able to maintain their weight on this diet for long periods.) As the curves for food consumption show, these animals took from 0.5 to 1 gm. of carrot proteins per day, and maintained body weights of 50 to 90 gm. respectively. This is equivalent to 10 gm. of protein per kilo of body weight, or about six times the amount allotted to an adult man on the basis of 120 gm. of protein for 70 kilos of body weight,—which is a high protein diet as human dietaries are usually reckoned. Similarly, as far as calories are concerned, these animals on an exclusive carrot diet

were consuming an amount of food which yielded 300 to 500 or more calories per kilo of body weight per day; whereas 50 calories per kilo per day for an adult man at moderately hard work, or 100 calories per kilo for the new born human infant, are considered liberal standards. Yet these rats were unable to make a growth upon this diet. Such facts lead us to conclude, that if we are to attempt to apply widely Rubner's laws of constant energy consumption and growth quotient (2) we must at the outset hedge about that generalization rather narrowly by defining the rate of growth to be expected and the quality as well as quantity of nutrients to be fed.

It has often been contended that since most feeding experiments with rats have been done by the use of dried and often of cooked materials, it is unreasonable to suppose that the processes of drying and ordinary cooking can prejudicially influence vitamine content or other nutritive values. Further consideration, however, makes it apparent that quantitative as well as qualitative experimentation is desirable. The fact that the animal maintains a normal growth curve and reproduction on a daily diet consisting, for example, of 10 gm. of bread and 5 gm. of heated dried milk (equivalent in bulk to 50 gm. of fresh milk), does not in itself prove that the cooking and drying have not in any way altered the nutritive value of milk. For it is conceivable (until the contrary has been proved) that the animal might have been as satisfactorily nourished on 10 gm. of bread, plus 25 gm. of fresh milk, instead of using the equivalent in dried form of twice that amount.

It is well recognized that temperatures considerably above 100°C., when applied for one or more hours, do prejudicially affect the nutritive values of some foods (3). Some investigators have concluded that ordinary cooking or even pasteurizing processes are liable to weaken or destroy the antiscorbutic or other valuable properties of milk or of vegetables, in some instances at least. We there ore considered that it would be of interest to ascertain whether carrots cooked after common methods would yield identical results with raw carrots, in the diet of white rats; *i.e.*, of an animal which is not usually considered as being subject to scurvy, so far as we know at the present time.

Methods of Experimentation.

In the first period of feeding, February 6 to March 12, the carrots were scraped or pared, sliced in sections ¾ to 1 inch thick; those fed to Lots I and II were boiled for 30 to 40 minutes in twice their own weight of distilled water. These diets were freshly cooked every other day, and stored in an ice box between the feedings, which occurred twice daily. They were fed without the addition of the juice, which had cooked out, to Lot I; also *with* the addition of this concentrated juice to Lot II. Calorimetric, Kjeldahl, and ash determinations showed an average extraction of about 20 per cent; nitrogen losses were somewhat irregular, being often considerably higher than the losses in salts and in fuel value. During the later period from March 12 up to the present date, when casein, starch, and butter had been added to the carrots, the time of cooking and the amount of water used were increased, and the extraction reached 25 to 30 per cent, so that it equalled that of the average of canned carrots described below.

Carrots were canned by the use of the cold pack method recommended for home canning by the Extension Service of the U. S. Department of Agriculture. This involves 5 minutes blanch of sliced carrots in boiling water, cold dip, packing into glass quart jars, and 2 hours process submerged in bath of boiling water. Our jars contained 700 gm. of carrots (weighed raw) and about 280 cc. of juice remained in the jar at the end of the process. The various tests used showed an extraction of 20 to 40 per cent in different jars and lots, varying with the amount of juice in the jar at the end of processing; average about 28 per cent. Evidently the lessened extraction due to the small amounts of water in the jar almost counterbalanced the tendency of blanching and long processing to increase the degree of extraction above that attained in the ordinary open kettle home cooking process. These canned carrots, after being stored from 3 to 6 months, were fed to Lot IV, Chart I.

We used commercial casein, soaked 1 week in slightly acidulated water (16 times its own weight, decanted off and renewed once each day), rinsed free of acid; twice extracted with 10 times its own weight of ether, which is decanted off; then spread on a

filter to dry. Leaf lard was rendered and strained through cheesecloth. Butter fat was heated in a water jacket and passed through filter paper. The starch was Kingsford's cornstarch.

In all cases animals were given as great a quantity of food as they could be induced to consume, and a record was kept of the food consumption of each animal.

Cooked Diets Compared to Raw.

It will be noted that the differences between growth curves of rats fed on raw carrots and those fed on cooked carrots with or without the juice, during the period of exclusive carrot plus salts diet (February 5 to March 12), are perceptible, though not large enough to be highly significant. However, the differences in amount of food consumed in order to make this growth are perhaps significant. The rats fed on cooked carrots without the extracted juice, soon developed a tendency to eat larger amounts of food than did the other two sets, and the same thing was true of the rats fed on canned carrots without the juice. On the other hand, rats fed on cooked carrots plus the juice extracted by cooking consumed somewhat less food than did the other two sets, even at the beginning. These differences in food consumption extended into the period when the carrots fed were fortified by addition of casein plus starch plus butter fat, becoming increasingly greater as time went on; *e.g.*, in 15 to 16 weeks after the beginning of the experiment, rats on raw carrots (fortified), were averaging about 100 gm. of food per day; those on cooked carrots (fortified) plus juice, about 80 gm. per day; those on cooked carrots (fortified) without the juice, 120 gm. per day; those on canned carrots (fortified) without juice, 100 to 110 gm. per day. These rats were in most cases from 150 to 220 gm. in weight, at that time.

During these later periods, percentages of extraction averaged 25 to 30 per cent for both boiled and canned carrots. In the case of the boiled carrots, the juice was more strongly concentrated than before; 3,500 gm. of water were measured out, in which to cook 1,500 gm. of carrots, and after boiling 1 hour the watery juice remaining in the kettle was then concentrated to 200 gm. by slow boiling in an open dish. This was about twice the de-

gree of concentration employed during the first 5 weeks of the feeding period, as far as final bulk of juice was concerned. The rats had shown themselves very fond of this earlier and less concentrated juice, taking it in preference even to the boiled carrots; but they did not like the more concentrated juice, and it had finally to be mixed with the carrots in order to insure their taking it at all. As it had a somewhat rank flavor and also more or less of the taste of caramel or burnt sugar, even when very carefully heated, these flavors were quite perceptible in the diet with which the juice was incorporated.

It is therefore possible to assign several reasons for the failure of the animals on the diet of boiled carrots plus juice, to eat as much food as did the other lots. First, due to some oversight, this lot contained two females and one male, whereas the other two lots contained two males and one female, therefore the three lots are not strictly comparable. It is believed, however, that the lower food consumption of Lot II is not due entirely to this fact. Second, the total bulk of food which must be taken in order to get the same number of calories or grams of protein, is greater in case of boiled carrots plus juice, than in the case of raw carrots, due to the addition of water absorbed by carrots in cooking, and to the water added to the juice which cooked out. It is true that rats are able to increase their food consumption to a remarkable degree as the food becomes more dilute, yet there are limits to their capacity in this direction. Third, their dislike of the strong flavor may have been a cause of gradually diminished appetite. Fourth, it is possible that during the extra period of long heating, especially on the concentrated juice, there was produced some chemical substance which had a depressing effect upon metabolism and therefore upon hunger and appetite. Fifth, it is difficult to explain either the difference in food consumption or that in growth curves as being due to destruction of vitamines by long boiling of the juice in concentrated form; for at the outset, when the quantities of food taken by Lot I, fed on boiled carrots without the addition of extracted juice, and by Lot II, who ate boiled carrots plus the juice, were about equal, the amounts of vitamine taken would have been about equal even if it were to be supposed that the vitamines extracted into the juice were entirely destroyed by heat.

Notwithstanding their larger food consumption, the animals of Lot I (boiled carrots without the juice) had greater difficulty in maintaining themselves in weight equilibrium than did those of Lot III, fed on raw carrots; and the same thing is true of the animals of Lot II (boiled carrots plus juice) and of Lot IV (canned carrots without the juice). This difference is apparent in the first period, when carrots were fed without fortification except by salts, but it is almost altogether lost in later periods, when casein, starch, and butter fat in varying amounts, fortified by a complete salt mixture, were added to the diet. Even when carrot solids formed one-half of the total solids of the diet, the effect of boiling was practically negligible, so far as any influence on the weight curves was concerned. Certainly the cooking of carrots used in human dietaries, when combined with other foods on any scale at present imaginable, cannot be supposed to have deleterious effects, so far as the results of these experiments are concerned. In saying this, we do not forget the interesting experimental work of the Norwegian investigators, Holst and Frölich (3), who report that they were unable to produce scurvy in rats, though animals subject to scurvy showed the deleterious effects of cooking upon antiscorbutic vegetables.

General Effects of Exclusive Carrot Diets.

All of the exclusive carrot diets produced a constant and considerable degree of diuresis. This diuresis was not altogether due to the large water content of the vegetable since some degree of diuresis was observed in animals fed on a diet of dried carrots. 30 to 45 gm. of urine were collected upon several occasions in 1 day, from a 200 gm. rat. Also the feces were greatly increased in amount and somewhat increased in water content; at times they showed large amounts of undigested matter, particularly in animals not yet accustomed to the diet, and at times there were periods of diarrhea. Rats of 150 gm. or over had much more difficulty when first introduced to the carrot diet, than did those of 40 to 90 gm. All animals during the earlier periods of exclusive carrot feeding showed the familiar symptoms of malnutrition, such as great restlessness, generally increased nervous irritability, emaciation, rough coat, and humped back

(muscular weakness or perhaps failure of muscles to keep pace with growth of bones). For some days or weeks also, they showed distended bellies, presumably due to distention by undigested carbohydrates or other residue, for there was no unusual accumulation of gas and no abdominal dropsy.

Two litters of rats, born of mothers living on low protein and carrot-rich diets, died at 2½ and 5 weeks respectively. It seemed that the high degree of diuresis shown by the mothers at that time might have prevented a proper flow of milk.

Nutritive Components of Carrots.

The carrots used in most of these experiments showed by usual Kjeldahl test about 1 per cent protein ($N \times 6.25$) on the basis of fresh weight (if not at all wilted), or an average of nearly 10 per cent on the basis of dry weight. It is believed to be the case that a large part of this nitrogen does not occur as protein, but that fact need not enter into the discussion here presented.

It is only under the most favorable conditions, and when carrots are fed without dilution of their protein by addition of starch or fat, that the carrot nitrogen is able to maintain body weight in equilibrium without the addition of foreign protein. When circumstances permit, however, not only does maintenance occur upon the exclusive carrots plus salt mixture diet, but there is even an interval between 3 and 8 weeks after the time feeding was started, when there may be a distinct tendency to fatten with increase of weight, although little or no growth of skeleton has occurred (see weight curves of No. 28, Chart II; first 5 weeks of Nos. 1, 10, and 12, Lot III, Chart I).

However, when the proportion of nitrogen in the diet was reduced by adding starch and fat to the carrots (fortified with salts but not with casein or any source of nitrogen), the animals are no longer able to maintain their body weight even for a period.

That carrots contain a not inconsiderable amount of both the water-soluble and the fat-soluble vitamines is evident from the growth curves of Lots I, II, III, and IV, Chart I, and Lots VII and VIII, Chart II. None of these diets contains any source of the water-soluble vitamine, outside of the carrots.[1] All of these

[1] We know this to be the case, for a set of control animals fed with these same lots of purified casein, starch, salts, and butter fat, made up into a

growth curves continue normal through the first generation. Reproduction took place in case of all females, as soon as they were put with the males. The young, however, are not making perfectly normal growth, and the cause of this is still under investigation. Variations in the nutritive values of carrots from different lots are also being studied.

Dropsy in Albino Rats as Induced by Carrot Diets.

The problem of diet as related to dropsy is one of greater importance and interest than ever before, since the appearance of "war dropsy" among the victims of malnutrition during the present war. It has been pretty generally ascribed to a lack of fat; but from our experience with albino rats, we are inclined to attach at least equal if not greater importance to the protein or nitrogen content of the diet.

In the mixtures used for feeding rats in Lots XV, XXXI, and XVI, Chart I, the carrot proteins ($N \times 6.25$) constituted between 4 and 5 per cent of the entire dry weight; instead of constituting about 10 per cent, as in the exclusive carrot diets.

Lot XVI was fed on the following diet. Raw chopped carrots, 375 gm.; cornstarch, 30 gm.; complete salt mixture, 3 gm.; lard, 5 gm. No. 76 died at the end of 3 weeks; no dropsy was observed. No. 68 died at the end of 5 weeks with a large amount of fluid in both peritoneal and thoracic cavities. No. 59 died at the end of $7\frac{1}{2}$ weeks. A large amount of fluid was collected subcutaneously on the chest several days preceding death; this fluid soon escaped freely, wetting the hair along the midventral

synthetic diet, exhibited in their growth curves satisfactory evidence of the absence of the water-soluble vitamine. We also tested another set of control animals with the starch, salts, lard, and purified casein, plus an alcoholic extract of wheat germ; although not all of the symptoms were obtained which we expected in absence of the fat-soluble vitamine (*e.g.*, edema of the eyes), yet there was a striking difference between the growth curves and general appearance of these rats, and those fed on a diet identical with it, except for the replacement of 5 per cent lard by 5 per cent butter fat. Except for a very small amount of fat-soluble vitamine which may be present in some of these purified foodstuffs used as fortifications, carrots are the only source of vitamine in Lots VII and VIII. Further investigations are being made on this point.

line; the amount of fluid found at autopsy was much above normal.

Lot XV was fed the same diet as above except that butter fat replaced lard. Of this lot No. 79 showed the same appearance at autopsy as No. 68 above. Nos. 55 and 75 died at the end of 8 weeks as a result of accident, but had shown no dropsy up to that time. Lot XXXI was fed the same diet as Lot XV above. No. 123 died at the end of 3 weeks, No. 130 at the end of $7\frac{1}{2}$ weeks, but no dropsy was observed in either case. No. 115 exhibited a remarkable case of dropsy; indeed the weight curve alone shows it most markedly; for there was an increase from 42 to 67 gm. in body weight within a few days before death. This increase was entirely due to the dropsy, as the animal had been steadily losing weight for some time. The day before death the right eye was seen to be badly swollen and almost closed. This swelling gradually spread over the entire body. At autopsy extensive collection of lymph was found under the skin of the legs and of the entire body, also in the thoracic and abdominal cavities.[2]

As yet we have had no true cases of dropsy in rats fed on carrots plus starch plus salts, without the addition of fat although such diets have been running for 10 weeks. In this lot Rats 85 and 86 died at the end of the 8th week. At no time was any marked swelling noticed in either, but in both there was a noticeable swelling of the eyelids a little more than a week before the time of death. From the time the swelling was first noticed, there was an escape of fluid which kept the hair around the mouth and midventral line of the body wet. At autopsy neither of these rats had an abnormal amount of fluid either subcutaneously or in the thoracic or peritoneal cavities, but judging from the rate at which the hair became wet, after being washed and dried, probably 1 or 2 cc. of fluid escaped in this way daily.

There have been no cases of dropsy on fresh, cooked, or dried carrots plus salts. This work on dropsy is very incomplete, but is still in progress and will be reported later.

[2] Autopsies and further study were given all these cases by Dr. H. Gideon Wells, of the Department of Pathology, who will doubtless report his findings in due time.

SUMMARY.

1. Ordinary methods of cooking do not perceptibly injure the nutritive value of carrots, or certainly not when used as part of a mixed diet. However, a considerable portion of the caloric value of the food is lost when the water used in cooking is rejected, as shown by the greater food consumption of animals on this diet, when the juice in which the carrots were boiled is not added to their food.

2. Carrots when properly supplemented with starch, purified commercial casein, butter or lard, and salts, to such an extent that 50 per cent of the caloric value of the diet is still derived from carrots, will produce normal growth and reproduction in albino rats.

3. Carrots as an exclusive diet, except for the addition of calcium, phosphorus, sodium, and chlorine, may support animals in apparently good health for as long as 16 weeks. Although there is no growth, the animals on this diet may maintain and at times even increase their own body weight. Due to a great variation in the nutritive value of carrots of different lots and in the vitality of different animals, results with this diet are somewhat inconstant.

4. Carrots have a considerable amount of both the water-soluble and fat-soluble vitamines.

5. Dropsy occurs in a large per cent of rats fed on a carrot diet, when the proportion of nitrogen has been reduced by the addition of some non-nitrogenous foodstuff, such as fat or starch.

BIBLIOGRAPHY.

1. Sherman, H. C., Food products, appendix E, New York, 1917. Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Office Exp. Stations, Bull. 28* (revised), 1906.
2. Rubner, M., Das Problem der Lebensdauer und seine Beziehungen zu Wachstum und Ernährung, Munich and Berlin, 1908.
3. Chick, H., and Hume, E. M., *J. Roy. Army Med. Corps*, 1917, xxix, 121. Frölich, T., *Z. Hyg.*, 1912, lxxii, 155. Funk, C., *J. Biol. Chem.*, 1916, xxv, 416. Funk, C., and Macallum, A. B., *ibid.*, xxvii, 61. Hogan, A. G., *ibid.*, 1917, xxx, 115. Holst, A., and Frölich, T., *Z. Hyg.*, 1912, lxxii, 1. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1918, xxxiii, 247. Osborne, T. B., and Mendel, L. B., *ibid.*, 1915, xx, 381.

Lot I
Boiled carrots, no juice
Lot II
Boiled carrots, with juice
Lot III
Raw carrots
Lot IV
Canned carrots
Period 1 2 3 4 5 6
Food consumption curves
Growth curves
Grams
200
150
100
50
250
200
150
100
50
Feb. Mar. Apr. May
5 12 9 10
Feb. 26 Mar. 12 Apr. 9 May 10

CHART I.

CHART I. Growth curves and food consumption curves. Rations for rats.

Period	1	2	3	4	5	6
Carrots, *gm.*...	1,000	1,000	1,000	1,000	1,000	750
Salt II, " ...	4.25	4.25	4.25	4.25	4.25	3.19
Salt III, " ...		3.7	3.7	3.7	3.7	3.8
Starch, " ...		48	24	12	12	37
Casein, " ...		24	12	24	24	24
Butter fat, *gm.*		20	10	10	10	10
Water for paste, *cc.*....		400	300	300	150	150

Salt Mixture II.

	gm.
Ca lactate	1.57
NaCl	0.5
$CaH_4(PO_4)_2 + H_2O$	0.6

Salt Mixture III.

	gm.
NaCl	0.173
Na_2SO_4	0.318
$NaH_2PO_4 + H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2 + H_2O$	0.540
Ca lactate	1.300
Fe lactate	0.118

Growth curves
Lot VII
Raw carrots
Lot VIII
Boiled carrots
Lot V
Lot XV
Lot XXXI
Lot XVI
Period
4 5 6
4 5 6
Apr. May
2 10
Apr. May
2 10
grams
250
200
150
100
50

CHART II.

CHART II. Showing growth curves of Lots VII, VIII, V, XV, XXXI, and XVI. Ration for Lots VII and VIII same as for Lots II and III, Chart I, at corresponding periods except that lard was used instead of butter. Ration for Lots V, XV, XXXI, and XVI as follows:

	Lot V.	Lots XV and XXXI.	Lot XVI.
Carrots, *gm*.....	100 (Dry.)	375 (Fresh.)	375 (Fresh.)
Salt II, "	4.25	1.6	1.6
Starch, "		30	30
Salt III, "		1.4	1.4
Fat, "		5 (Butter.)	5 (Lard.)
Water, *cc*.....		75	75

CREATINURIA. I.

EXOGENOUS ORIGIN OF URINARY CREATINE.*

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In recent years there is probably no problem of intermediary metabolism in animals that has occupied the attention of, and been subjected to experimental investigations by physiological chemists as much as that of creatine and creatinine. In part this is due to the excellent work of Folin who has made a quantitative determination of these compounds possible, but to a considerable extent it is also due to the apparently many interesting and puzzling relations of these compounds in their origin and fate in the animal body in health and in disease.

It is not intended to present here a lengthy résumé of the general progress that has been made in this field except in so far as in justice to others it becomes imperative to point out in what respects our points of view agree with, or differ from those held by others. In this paper we shall present some of the factors which we believe to be responsible for the apparent anomalies presented by the appearance and disappearance of creatine in the urine under different dietary conditions.

Fasting.

Creatine was first observed to be a constituent of the urine of fasting man by Benedict[1] and Cathcart.[2] Dorner[3] made the

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Benedict, F. G., *Carnegie Institution of Washington, Publication 77*, 1907.

[2] Cathcart, E. P., *J. Physiol.*, 1906-07, xxxv, 500.

[3] Dorner, G., *Z. physiol. Chem.*, 1907, lii, 225.

same observation on fasting rabbits. Since then Richards and Wallace,[4] Underhill and Kleiner,[5] Howe and Hawk,[6] and others have obtained corresponding results with dogs, but McCollum and Steenbock,[7] in 1912, found that the pig fasting for as long a period as 14 to 16 days did not excrete creatine. This surprising observation led to the formulation of the thesis that the pig, in comparison with most other animals having greater capacity to store fat, might be possessed of greater ability to use fat during emergencies and thus conserve such other body tissues as would liberate creatine in their disintegration. In an attempt to confirm these observations and establish this hypothesis with additional data we have been surprised to find that the failure of the pig to excrete creatine during fasting, at least with the animals from the University herd, is the exception rather than the rule. To establish the reason for these differences our investigations on creatine excretion have become rather more extensive than originally planned.

Mendel and Rose[8] in studying creatinuria in the rabbit also observed a difference in the behavior of animals with respect to the ease with which creatinuria was induced. One rabbit in an exceptionally good nutritive condition had creatine in its urine in more than traces only as the period of fasting was exceptionally protracted. It is evident that with careful control of the diet or even withholding of all foods there obtain conditions in the apparently normal animal which determine the presence or absence of creatine in the urine.

Cathcart[9] reported that creatine excretion induced in men by fasting could be reduced to nil by the administration of a diet practically nitrogen- and fat-free. Mendel and Rose[8] criticized Cathcart's results as the diet employed by him was far from having an insignificant nitrogen content, but with the diet absolutely free from nitrogen and fats they obtained with rabbits results the same as Cathcart's.

[4] Richards, A. N., and Wallace, G. B., *J. Biol. Chem.*, 1908, iv, 179.
[5] Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 167.
[6] Howe, P. E., and Hawk, P. B., *J. Am. Chem. Soc.*, 1911, xxxiii, 215.
[7] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.
[8] Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213.
[9] Cathcart, E. P., *J. Physiol.*, 1909–10, xxxix, 311.

That carbohydrate feeding in many instances is able to reduce the amount or even prevent the appearance of creatine induced by fasting is brought out in Table I in our experiments with a pig. A pig was confined in a metabolism cage and 24 hour collections of urine were made. Nitrogen was determined by the Kjeldahl method, and creatine and creatinine by Folin's original method using Benedict's method of dehydration for the creatine. The picric acid used was found to be sufficiently pure to preclude the possibility of introducing an error greater than is ordinarily unavoidable in the method. The analyses were made daily, chloroform and toluene being used as the preservatives.

TABLE I.

Pig, male, weight 23 kilos. Fasted 3 days before collections were made.

Day of collection.	Urinary N.	Preformed creatinine.	Total creatinine.	Creatine as creatinine.	Diet.
	gm.	*gm.*	*gm.*	*gm.*	
1	5.16	0.425	0.708	0.283	Fasting.
2	6.00	0.302	0.812	0.509	"
3	5.24	0.447	0.836	0.388	"
4	3.66*	0.330*	0.564*	0.234*	"
5	3.51	0.372	0.766	0.422	Starch 500 gm.
6	3.16	0.506	0.581	0.075	" 500 "
7	1.91	0.490	0.490	0.000	" 500 "
8	1.87	0.596	0.679	0.083	" 500 "

* Incomplete collection.

As is seen in Table I the ingestion of 500 gm. of starch daily soon resulted in a marked reduction in creatine elimination. Concomitantly there is observed a reduction in nitrogen excretion. The feeding of carbohydrate may have a very pronounced effect on creatine excretion in fasting as indicated above, but this is not necessarily always the case for reasons indicated later. With the pig here under observation the nitrogen of the urine at the beginning of fasting was exceptionally high. This may have accentuated the observed favorable influence of carbohydrate feeding.

Acidosis.

Underhill and coworkers,[10] in 1916, on the premises stated by them, "that in nearly every instance in which creatine appears in the urine there is an accompanying acidosis suggests the hypothesis that a condition of acidosis in the body is responsible for the appearance of creatine in the urine," carried out a series of experiments with the rabbit. They concluded "that there is an interrelationship between acidosis and creatine elimination. Creatine in the urine may prove to be an index of a condition of acidosis in the organism." In a later paper they bring out the fact that acidosis is not the only factor responsible for creatinuria as in the phlorhizinized dog administration of sodium bicarbonate leading to the voiding of alkaline urines was without appreciable influence upon the elimination of creatine. Again they state "creatine may appear in the urine in states of acidosis when carbohydrate deficiency is not involved and creatinuria may be present during carbohydrate deficiency even in the absence of acidosis."

That creatine elimination when occurring in the pig may be dependent at least in part upon an acidosis is brought out in Table II. A fasting pig was given by sound 30 gm. of sodium acetate, dissolved in water, in two portions on 2 successive days. The urine turned alkaline to litmus and the creatine as well as the total nitrogen was immediately reduced. Such data were obtained in many experiments, but as acidosis is not the sole determinant of creatinuria any more than carbohydrate deficiency they will not be presented here *in toto*.

Although creatinuria induced by fasting in the pig can be reduced in degree or even prevented by carbohydrate feeding as well as by the ingestion of virtually or potentially alkaline salts, yet it need not necessarily be prevented by a combination of the two. This is brought out in Table III.

As is observed in Table III the creatine in the urine, while reduced in amount by the ingestion of both carbohydrates and alkalies, is not entirely removed. As the pig was getting a great excess of carbohydrate over that necessary to cover his energy

[10] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127, 141. Underhill, F. P., and Baumann, E. J., *ibid.*, 147, 151. Underhill, F. P., and Bogert, L. J., *ibid.*, 161.

requirements, and a sufficient amount of sodium acetate to make his urine strongly alkaline to phenolphthalein, certainly then, neither acidosis nor carbohydrate deficiency could be said to be the determinants of creatinuria. With a lower plane of creatine

TABLE II.

Pig, male, weight 25 kilos. Fasted 3 days before collections were made.

Day of collection.	Urinary N.	Preformed creatinine.	Total creatinine.	Creatine as creatinine.	Diet.
	gm.	gm.	gm.	gm.	
1	11.38	0.312	1.311	0.999	Fasting.
2	9.72	0.318	0.991	0.673	"
3	4.16	0.324	0.469	0.145	Sodium acetate 30 gm.
4	4.13	0.383	0.501	0.118	" " 30 "

TABLE III.

Pig, male, weight 27 kilos. Fasted 2 days before collections were made.

Day of collection.	Volume of urine.	Urinary N.	Reaction of urine.	Ammonia N.	Preformed creatinine.	Total creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.		gm.	gm.	gm.	gm.	
1	675	11.41	Acid.*	0.935	0.574	0.957	0.382	Fasting.
2	450	8.25	" *	0.603	0.438	0.877	0.438	"
3	360	8.08	" *	0.817	0.434	0.852	0.417	"
4	600	7.06	Alkaline.†	0.115	0.648	0.879	0.231	Starch 250 gm. Na acetate 30 gm.
5	1,625	3.94	" †	0.248	0.603	0.774	0.171	Starch 500 gm. Na acetate 20 gm.
6	2,200	3.22	" †	0.267	0.509	0.774	0.264	Starch 500 gm. Na acetate 20 gm.
7	1,750	3.54	" †	0.148	0.653	0.854	0.200	Starch 500 gm. Na acetate 20 gm.

* To litmus.

† To phenolphthalein.

elimination from that observed in this experimental animal we have every reason to believe that instances may occur where reduction of the acidosis or removal of carbohydrate fasting singly or jointly may lead to the total disappearance of the creat-

inuria. It should be emphasized that here again the reduction of the creatinuria was accompanied by a marked decrease in the nitrogen excretion.

In an experiment carried out previous to those already cited we had made the attempt in a fasting pig, which failed to show

TABLE IV.

Pig, male, weight 35 kilos. Collections started on the 5th day of fasting.

Day of collection.	Volume of urine.	Urinary N.	Acidity of 25 cc. urine. Cc. N/14 NaOH.	Ammonia N.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	*gm.*		*gm.*	*gm.*	*gm.*	
1	1,260	3.57	5.7	0.257	0.833	0.000	Water.
2	1,440	2.88	4.1	0.201	0.761	0.000	"
3	670	2.76	10.1	0.201	0.798	0.000	"
4	700	2.67	6.7	0.145	0.833	0.000	"
5	1,405	3.06	3.0	0.320	0.759	0.000	" +2.5 cc. C.P. HCl.
6	1,350	2.54	3.5	0.459	0.624	0.000	"
7	1,490	3.22	4.3	0.643	0.946	0.000	" + 10 cc. C.P. HCl.
8	1,440	2.37	3.4	0.869	0.864	0.000	"
9	475	1.41	6.3	0.249	0.499	0.000	" + 10 cc. C.P. HCl.*
10	1,530	3.39	2.9	0.881	0.991	0.000	"
11	1,385	1.95	2.6	0.426	0.669	0.000	"
12	1,470	1.75	2.2	0.352	0.744	0.000	"
13	1,537	1.94	2.0	0.325	0.748	0.000	"
14	1,460	2.47	2.7	0.671	0.705	0.000	" + 10 cc. C.P. HCl.
15	1,585	2.85	3.5	1.109	0.703	0.000	" + 10 " " " HCl.
16	1,450	2.51	2.4	0.986	0.700	0.000	" + 5 " " " HCl.*
17	1,535	2.41	2.5	0.749	0.656	0.000	"
18	1,545	2.16	2.0	0.568	0.625	0.000	"
19	1,515	2.06	2.1	0.412	0.613	0.000	"

* Part of acid lost by vomiting soon after administration. On the 16th day of collection at least one-half of the acid was lost in this way, but collections of urine were not contaminated.

creatinuria, to produce creatinuria by the administration of an inorganic acid. That our attempts were entirely unsuccessful, even though 5 and 10 cc. of concentrated hydrochloric acid were given on successive days, is shown in Table IV.

The failure to induce creatinuria in this animal led us to attempt to repeat the experiment. We were, however, unable to

find another pig which did not have creatine in its urine, so that our endeavors were necessarily confined to attempts to increase the creatine excretion of animals already excreting creatine in the urine. In the experiment presented in Table IV we were impressed with the difficulty of producing a strongly acid-reacting urine even when as much as 10 cc. of concentrated hydrochloric acid were given to the fasting animal. It is true that our only measure of the acidity of the urine was a titrimetric measure of its alkali-absorbing capacity rather than its hydrogen ion concentration, but in a rough way under many biological conditions such values have been shown to run sufficiently parallel for comparative results.[11] For determination of the degree of acidity we have in later experiments determined the hydrogen ion concentration of the urine by the colorimetric method using Clark's standards.[12] In all cases the acidosis induced by acid administration was but small as compared with the values on rabbits obtained by Underhill.[10] We secured additional evidence of the tendency to an acidosis, however, by the tremendous increase in the values for ammonia. In some cases we also followed the phosphorus excretions.

As seen in Table V, upon the administration of 5 cc. of concentrated hydrochloric acid on 2 days the hydrogen ion concentration was increased slightly, the ammonia increased greatly, yet the creatine did not increase; in fact, on 1 day after the acid was given the creatine disappeared entirely. With this animal the amount of acid given could not be increased as such attempts led to immediate regurgitation of stomach contents.

In Table VI a pig was given 1.53 gm. of hydrochloric acid diluted to 1,200 cc. with distilled water. The acid was given with a sound in four portions during the course of the day. The acid was traced to the urine by the observed increased chloride excretion as determined by the Volhard-Arnold method. When acid was given there was a general increase in the hydrogen ion concentration of the urine although the effect was not observed immediately from day to day. On 3 out of the 4 days when acid was given there was a decided increase in creatine elimina-

[11] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1914, xvii, 305.
[12] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917 ii, 109, 191.

TABLE V.

Pig, male, weight 36 kilos. Collections started on the 8th day of feeding the starch diet.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	pH	Creatinine.	Creatine.	Diet.
	cc.	gm.	gm.		gm.	gm.	
1	3,200	1.86	0.193	6.4	0.649	0.191	Starch 300 gm.
2	2,150	1.59	0.189	6.4	0.731	0.137	" 300 "
3	1,750	1.53	0.257	6.4	0.901	0.130	" 300 "
4	1,850	1.85	0.740	6.0	0.889	0.147	" 500 " + 5 cc. C.P. HCl.
5	1,700	1.70	0.605	6.4	0.892	0.000	" 500 "
6	1,900	2.01	0.866	6.0	0.836	0.114	" 500 " + 5 cc. C.P. HCl.
7	1,375	1.86	0.825	6.2	0.896	0.101	" 500 "

TABLE VI.

Pig, male, weight 18 kilos. Fed on 350 gm. of starch daily 5 days before collections were made, but on the first 2 days food was refused, on the next 2 days it was partly consumed, and only on the last day just previous to the beginning of the record it was all consumed.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	pH	Creatinine.	Creatine as creatinine.	Chlorides as HCl.	P_2O_5	Diet.
	cc.	gm.	gm.		gm.	gm.	gm.	gm.	
1	510	1.63	0.414	7.4	0.327	0.032	0.01	0.54	Starch.
2	550	1.56	0.295	7.2	0.396	0.035	0.09	0.33	"
3	1,910	1.86	0.609	6.3	0.399	0.130	1.04	0.32	" + HCl 1.53 gm.
4	500	1.37	0.378	6.5	0.398	0.035	0.62	0.30	"
5	1,675	2.45	0.610	6.5	0.399	0.121	1.29	0.61	" + HCl 1.47 gm.
6	450	1.90	0.521	6.3	0.455	0.065	0.53	0.53	"
7	310	1.47	0.329	6.1	0.448	0.045	0.13	0.53	"
8	1,600	1.83	0.576	6.5	0.483	0.078	0.78	0.54	" + HCl 1.53 gm.
9	1,600	1.88	0.650	5.9	0.385	0.115	1.40	0.61	" + HCl 1.53 "
10	370	1.49	0.532	5.8	0.457	0.035	0.56	0.38	"
11	310	1.88	0.444	6.0*	0.405	0.100	0.35	0.46	"

* On the 2 following days as the starch diet was continued the pH had come back to 6.5 and 6.4 respectively.

tion. This indicates that acid administration, even in the pig with its tremendous capacity for counteracting the tendency to establish acidosis, can cause an increase in creatine excretion. During such times when the creatine did not increase it may be surmised that the acid-neutralizing mechanism may have been so efficient that the threshold for increased creatine production was not crossed. Weight is given to this point of view by the fact that on such days the hydrogen ion concentration of the urine was not very high. Our data are not of the character to establish this definitely, but nevertheless we believe them to be strongly suggestive of this point of view. Whether by virtue of the comparatively slight increase in hydrogen ion concentration or the great increase in ammonia excretion, we are willing to grant that an acidosis obtained; the fact nevertheless is clear that acid administration to the extent that it is tolerated by the animal may or may not increase creatine elimination. Acidosis and carbohydrate deficiency singly or jointly play a rôle in certain forms of creatinuria.

Protein Feeding.

In 1911, when Mendel and Rose[8] confirmed Cathcart's[9] observations that creatinuria may be prevented by carbohydrate feeding, they also stated that, "The metabolism of exogenous or reserve proteins is not accompanied by the production of creatine or creatinine." Just why they should reach this conclusion is not quite clear as their experimental data were not of the character to bring out this point conclusively. In 1912, Folin and Denis[13] in verifying Rose's findings that creatine is usually present in the urine of children, even on a creatine-free diet, stated: "We are inclined to believe that the creatine in children's urine does not depend . . . . on a peculiar carbohydrate metabolism but that it is due to an excessively high level of protein consumption (in proportion to mass of muscles in the body)." Furthermore, they said, "If the above hypothesis is correct it should be possible to reproduce in adults by forced feeding with protein which contains no creatine the conditions with reference

[13] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 253.

to creatine found in children and it should also be possible to obtain creatine free urine from children by reducing their protein consumption." Shortly afterwards it was shown in this laboratory[7] that with the pig ingestion of protein from different sources could lead to decided differences in creatine excretion. The kind as well as the amount of protein ingested seemed to be important in this connection. Powis and Raper[14] concluded: "Assimilation of food has little or no influence on creatine excretion." An increased creatine excretion during the day as compared with that during the night was attributed by them to differences in state of rest of the skeletal muscles. Denis[15] was, however, unable to confirm their results. She found the creatine excretion highest after heavy meals and therefore still adhered to the theory of exogenous origin of creatine. Later Denis and Kramer[16] proved one of the points which Folin and Denis[13] had postulated as being necessary to establish the exogenous origin of creatine. They expressed themselves as follows: "We have found that by feeding children diets extremely low in protein but of sufficient calorific value, it is possible to obtain urines absolutely creatine-free. On feeding increasing amounts of protein, creatine excretion begins, increases day by day, and if the protein intake can be pushed to a sufficiently high point may become considerably greater than the excretion of preformed creatinine." Denis and Minot[17] subsequently also satisfied the other requisite demanded by the theory; that is, they were able to produce creatinuria in adults by feeding a high protein diet. It should, however, be mentioned that normal men in contrast with women presented a peculiar problem in that creatinuria has not yet been induced in them by high protein feeding. It has, however, been induced in a man afflicted with hyperthyroid activity. We shall discuss this later.

In spite of the evidence cited above, Rose, Dimmitt, and Bartlett,[18] while this paper was in preparation, have stated

[14] Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 362.

[15] Denis, W., *J. Biol. Chem.*, 1917, xxix, 447.

[16] Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.

[17] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1917, xxxi, 561.

[18] Rose, W. C., Dimmitt, J. S., and Bartlett, H. L., *J. Biol. Chem.*, 1918, xxxiv, 601.

"that the theory which attributes the excretion of creatine on a creatine-free diet to an exogenous origin in the protein of the diet is untenable." Apparently they arrived at this sweeping conclusion on the basis of their experiments in which they were unable by high protein feeding to produce creatinuria in men or to increase it in women.

In the experiments which follow we shall indicate again that creatine in the urine can be caused to appear or if present can be caused to increase in amount as the result of no other modification in the diet than the introduction of creatine-free protein. In Table VII we have a continuation of a record of performance of a pig fasted for 23 days, as tabulated in Table IV. It was fed 200 gm. of casein on the 1st day and 400 gm. for 4 consecutive days thereafter. It was then fasted, water always being given *ad libitum*. This record indicates with what remarkable persistence the absence of creatine may be maintained in spite of the long preliminary fasting period with acid ingestion. The final appearance of creatine with the ingestion of abnormally large amounts of protein, as well as its subsequent rapid disappearance when protein feeding was discontinued, is perfectly definite, and scarcely leaves room for the argument that the very resistance to inception of creatinuria by protein feeding should speak against high protein as a major factor in creatinuria production. In the case of this animal there is no doubt that smaller amounts of protein might have been fed even for a considerable time without the appearance of any creatine.

The situation was entirely different with the animal recorded in Table VIII. This pig which normally had enormous quantities of creatine in its urine doubled the excretion of its creatine when it was given only 200 gm. of casein. It may be stated that whenever creatine was already being excreted by an animal the amount of protein necessary to produce a certain amount of creatine was far less than when it was originally absent. In speaking of creatine production it should not be forgotten that the amount of creatine produced is undoubtedly not synonymous with the amount excreted.

In the experiments detailed in Tables VII and VIII it was possible that the creatine elimination was indirectly due to the stimulation of endogenous metabolism resulting from the sulfuric

and phosphoric acids produced in the metabolism of the casein. To determine the rôle of these factors we next gave a number of

TABLE VII.

Same pig used as for Table IV, the collections being continuous. Weight of animal 28 kilos.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	
20	1,100	3.20	0.318	0.667	0.000	Casein 200 gm.
21	1,180	12.44	0.900	0.820	0.000	" 400 "
22	1,430	21.90	1.779	0.780	0.095	" 400 "
23	1,910	33.62	2.493	0.799	0.370	" 400 "
24	1,925	35.37	2.701	0.715	0.683	" 400 "
25	1,700	32.55	2.318	0.678	0.249	Fasted.
26	1,130	16.19	1.056	0.695	0.051	"
27	1,700	7.00	0.520	0.791	0.000	"
28	1,415	3.18	0.331	0.736	0.000	"

TABLE VIII.

Pig, male, weight 24 kilos. Previous to the beginning of the collections recorded in this protocol the pig fasted for 8 days, on one of which, 4 days before the collections tabulated below were made, it received 30 gm. of sodium acetate.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to litmus.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	*gm.*	*gm.*		*gm.*	*gm.*	
1	1,250	4.83	0.256	Slightly alkaline.	0.460	0.481	Fasting.
2	1,640	4.41	0.271	" "	0.563	0.429	"
3	1,925	4.05	0.309	" "	0.482	0.539	"
4	2,675	8.14	0.480	" " *	0.424	0.924	Casein 200 gm.
5	3,500	14.78	0.464	" "	0.328	1.094	Fasting.
6	1,400	5.46	0.403	" "	0.333	0.344	"
7	2,700	6.96	0.208	" "	0.307	0.614	"
8	2,200	3.94	0.506	Neutral.	0.321	0.524	"

* Almost neutral.

pigs an amount of sodium acetate sufficient to keep their urine strongly alkaline, especially on the days when the casein was fed. The data of such experiments are recorded in Tables IX, X, and

XI. They were obtained on the same animals as those already presented in Tables I, II, and III respectively.

That the rôle of inorganic acid having its origin in the metabolism of exogenous protein is a minor or even a negligible one in creatine production, we have proved to our satisfaction in still another way. It was found that while protein feeding would cause an increase in creatine excretion, amounts of hydrochloric acid equivalent to the potential acidity of the sulfur of the in-

TABLE IX.

Same pig used as for Table I. After the completion of that record the starch diet was continued the following 5 days before the collections tabulated below were made. On the 2 days immediately previous to this record 20 gm. of sodium acetate were given daily.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to phenolphthalein.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	gm.	gm.		gm.	gm.	
1	1,820	1.33	0.047	Alkaline.	0.569	0.142	Starch 500 gm. Na acetate 20 gm.
2	1,450	1.82	0.021	"	0.512	0.239	Starch 500 gm. Na acetate 20 gm.
3	1,025	3.97	0.120	"	0.520	0.546	Starch 500 gm. Na acetate 22.5 gm. Casein 100 gm.
4	1,250	3.21	0.214	Acid.*	0.517	0.169	Starch 500 gm. Na acetate 17.5 gm.
5	1,350	3.25	0.071	Alkaline.	0.514	0.319	Starch 500 gm. Na acetate 20 gm.

* Alkaline to litmus.

gested protein were entirely ineffective. Needless to say, these experiments in comparative efficacy of protein and its acidity in creatine production had to be carried out on the same animals and furthermore could only be demonstrated with animals already excreting creatine. With others such as those of which the records were presented in Tables IV and VI the amount of protein necessary to produce creatinuria is so large that when attempts were made to give equivalent amounts of acid, vomiting resulted.

TABLE X.

Same pig used as for Table II. Only 1 day intervened between the two records, during which there was no change in diet.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to phenolphthalein.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	*gm.*	*gm.*		*gm.*	*gm.*	
1	1,750	2.81	—	Alkaline.	0.548	0.208	Starch 500 gm. Na acetate 30 gm.
2	1,550	1.69	0.067	"	0.604	0.212	Starch 500 gm. Na acetate 30 gm.
3	1,400	5.75	0.154	"	0.623	0.811	Starch 500 gm. Na acetate 50 gm. Casein 200 gm.
4	1,720	7.29	0.152	"	0.573	0.643	Starch 500 gm. Na acetate 40 gm.
5	1,500	3.58	0.063	"	0.770	0.137	Starch 500 gm. Na acetate 30 gm.
6	1,500	2.17	0.064	"	0.787	0.169	Starch 500 gm. Na acetate 30 gm.

TABLE XI.

Same pig used as for Table III. The first 2 days of this record are taken from Table III to bring out the effect of casein feeding on the creatine increase.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Reaction to phenolphthalein.	Creatinine.	Creatine as creatinine.	Diet.
	cc.	*gm.*	*gm.*		*gm.*	*gm.*	
1	2,200	3.23	0.268	Alkaline.	0.509	0.265	Starch 500 gm. Na acetate 20 gm.
2	1,750	3.54	0.148	"	0.654	0.200	Starch 500 gm. Na acetate 20 gm.
3	1,750	8.96	0.169	"	0.642	0.578	Starch 500 gm. Na acetate 30 gm. Casein 100 gm.
4	1,620	4.25	0.232	"	0.602	0.128	Starch 500 gm. Na acetate 30 gm.
5	1,800	3.61	0.154	"	0.640	0.164	Starch 500 gm. Na acetate 20 gm.

In the calculations, sulfur was assumed to be completely oxidized to sulfuric acid. The results of one such experiment are presented in Table XII.

TABLE XII.

Pig, male, weight 13.6 kilos. The pig was fasted for 3 days. It then ate 250 gm. of starch in the form of a paste daily for 6 days, on one of which, 2 days before the collections tabulated below were made, it received 0.75 gm. of HCl in addition. 600 cc. of distilled water with or without the acids were given daily by means of a sound.

Day of collection.	Volume of urine.	Urinary N.	Ammonia N.	Creatinine.	Creatine as creatinine.	Chlorides as HCl.	P_2O_5	Diet.
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	
1	1,000	0.98	0.316	0.286	0.055	0.538	0.279	Starch 250 gm.
2	1,000	0.73	0.203	0.270	0.081	0.359	0.233	" 250 "
3	1,000	0.78	0.298	0.289	0.048	1.172	0.178	" 250 " HCl 0.76 gm.
4	1,000	0.84	0.497	0.307	0.052	1.192	0.189	Starch 250 gm. HCl 1.52 gm.
5	700	1.28	0.529	0.317	0.052	0.414	0.348	Starch 250 gm.
6	650	4.69	0.575	0.308	0.161	0.652	0.322	" 158 " Edestin 83.3 gm.*
7	1,025	3.99	0.504	0.345	0.620	0.433	0.378	Starch 250 gm.
8	950	1.96	0.369	0.313	0.074	0.348	0.258	" 250 "

* The edestin was not a pure preparation, but contained some carbohydrate which reduced its N to 14.55 per cent and its S to 0.71 per cent. Calculated over into H_2SO_4 its S would be equivalent to 1.31 gm. of HCl in acidity.

DISCUSSION.

We have now demonstrated to our satisfaction that in the pig, creatine in the urine may or may not be produced by fasting. From data previously presented as well as from later occasional observations we have noticed that animals giving every external appearance of being normal may or may not have creatine in the urine on such rations as it is customary to feed in good animal husbandry practice. We have also shown that the creatinuria obtaining during fasting may be reduced by carbohydrate feeding or by the administration of alkali, but that both treatments applied simultaneously need not necessarily prevent it.

In addition, we are convinced that acid administration resulting in a slight acidosis may or may not increase the creatine, and that protein feeding, if sufficiently intensive, will always produce creatinuria or if already present will increase it in degree. This effect obtains even during alkalosis and cannot be attributed to acids resulting from the metabolism of the protein molecule.

We realize fully that our conclusions are at variance with many that have been made on an apparently unimpeachable experimental basis and we realize also that in the contentions here presented it may appear as though confusion in the study of creatine metabolism has been increased rather than diminished. On critical analysis this shows itself to be far from the case. We are convinced that in one form or another creatinuria is etiologically related to protein metabolism, whether of exogenous or endogenous origin, and that in addition, in a manner as yet unknown, it is related to the creatine stored in the muscles and other tissues as well. With this fundamental conception it becomes possible to correlate all reliable observations hitherto made on creatine excretion.

Since Folin,[19] in 1906, showed that creatine ingested by man did not often make its appearance in the urine or even increase the nitrogen excretion, the idea has been quite prevalent that ingested creatine is absorbed by the muscles and that creatinuria is then related to the degree to which saturation of the muscle obtains. Thus Denis[20] in discussing the effect of high protein diets stated: "It is exceedingly improbable, however, that any creatine excretion would result were the diet given to these hyperthyroid patients eaten by a normal woman. It would therefore seem possible that in this condition and in some other pathological conditions, as well as in childhood, the saturation point of the muscle for creatine is low, a hypothesis supported by the low values for muscle creatine found in children and in persons suffering from exophthalmic goiter." We can hardly see the force of this argument though we readily accept the conception of creatine storage and consider that it probably is a misstatement as Denis previously stated in the same article: "As a working hypothesis it may be suggested, therefore, that on the ingestion

[19] Folin, O., Festschrift Olof Hammarsten, Upsala, 1906, paper iii

[20] Denis, *J. Biol. Chem.*, 1917, xxx, 47.

of protein some fraction of this is transformed into creatine, transported to the muscles, and there absorbed. If so much creatine is manufactured that the muscles become supersaturated, creatine is excreted by way of the kidney." Again, Rose and coworkers[18] state: "Accordingly, the creatine excretion in children, and in adults with exophthalmic goiter may be due not only to the high protein of the diet, but perhaps also to a low saturation point of the muscle for creatine."

The creatine content of the muscle in its relation to creatinuria obtaining as it often does over long periods of time must be secondary in importance. While a certain amount of creatine found in the urine can have its origin in the creatine of the tissues, in the final analysis of creatinuria we must consider from what source of materials even that creatine was originally formed. Reduced to its simplest terms the present immediate problem resolves itself into a consideration of the mechanism of the formation of all creatine, as in the light of present experimental data we are not warranted in assuming that the creatine of the muscles owes its origin to different metabolic processes from that of the creatine excreted in the urine.

Creatine is formed from precursors in the protein molecule. Whenever the protein molecule is disintegrated, these precursors are liberated and to the extent that they are not utilized in the body an attempt is made to utilize their energy. In part, this results in the formation of urea but, as with the guanidine residue in arginine, urea formation to the exclusion of the formation of other nitrogenous decomposition products is not possible. Arginine may be catabolized by two essentially different processes. It may be split into urea and ornithine, which ultimately is also deaminated and excreted as urea, or it may be split into components leaving the guanidine grouping intact. In the latter instance cleavage would occur at the linkage of the carbon chain with the imino group, or the carbon chain would be deaminated and then oxidized to a two-carbon residue,—acetic acid. In the one case guanidine would be formed which would be acetylated—as guanidine is exceedingly toxic, while guanidine acetic acid is practically innocuous; in the other case we would already have guanidine acetic acid; in either case methylation would result in the formation of creatine. The biological methylation of guani-

dine acetic acid has been demonstrated experimentally,[3,21] so that this step offers no difficulty for conception, but we are not prepared to say by which of the two methods the formation of guanidine acetic acid occurs. In the *intra vitam* synthesis of creatine Henderson's[22] observations are of interest. She found that in parathyroidectomized dogs a decrease in the free guanidine of the muscle tissue corresponded well with an observed increase in creatine. At any rate, according to our hypothesis creatine is an end-product in the catabolism of certain precursors in the protein molecule just as urea is of others.

The above conception may be challenged on the basis of data pointing to the great variability in the amount of creatine excreted under different conditions and especially on account of the lack of a definite ratio between the total nitrogen and the creatine nitrogen. Howe and Hawk[6] stated: "That there is a direct relation between the amount of nitrogenous material catabolized and the creatine excreted is brought out in our results." But later[23] they point out the discrepancy in the amount of nitrogen eliminated and the creatine nitrogen if referable to complete muscular disintegration. In proportion to creatine nitrogen the total nitrogen should have been higher. Benedict and Osterberg[24] stated: "There is no parallelism between body tissue destroyed, and creatine elimination. Sparing the body nitrogen does not affect the creatine output." And again they[24] may be quoted: "Myers and Fine have recently suggested that the reason carbohydrate is effective in causing cessation of creatine elimination is because of the sparing action on protein metabolism. Such an explanation would be more satisfactory were it not that the creatine wholly disappears upon a pure carbohydrate diet, while only a portion of the muscle tissue can be spared by such a diet." M. S. Rose[25] also found that in women, creatine elimination in the urine is not definitely influenced by protein in the diet.

[21] Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430.

[22] Henderson, P. S., *J. Physiol.*, 1918, lii, 1.

[23] Howe, P. E., Mattill, H. A., and Hawk, P. B., *J. Biol. Chem.*, 1911–12, x, 417.

[24] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1914, xviii, 195.

[25] Rose, M. S., *J. Biol. Chem.*, 1917, xxxii, 1.

We believe that the above arguments can be successfully answered from information available as a result of modern investigations on protein metabolism, keeping in mind the possibility that the metabolism of creatine precursors may proceed in different directions and that the creatine precursors during maintenance, but especially during growth, during body tissue restitution, and during milk production are utilized and not completely metabolized to creatine.

It is significant that creatine generally occurs in the urine in instances where there is an increased protein catabolism; *viz.*, during fevers, in exophthalmic goiter, in tumor cachexia, in post partum resolution of the uterus, in hydrazine and phosphorus poisoning, and in diabetes mellitus. It is also significant that creatine in the urine is reduced or prevented by just those agents which reduce protein catabolism. For instance, carbohydrates and alkaline salts both reduce protein catabolism and both reduce creatinuria; fats do not reduce protein catabolism and correspondingly do not reduce creatinuria. With respect to carbohydrates this conception is not new, as Shaffer and Coleman[26] in 1909 found that diets rich in carbohydrates in the treatment of typhoid prevented the loss of creatine or reduced it to a minimum which they attributed to a body protein-sparing action. Myers and Fine[27] in 1913 stated: "The decreased elimination of creatine after feeding carbohydrate is primarily dependent upon the sparing action of carbohydrate . . . . on protein metabolism." But further than this they did not go.

With protein feeding the matter is apparently far different, but actually it is analogous. It becomes a question of supplementation of amino-acids in such a manner that after the demands of the body are satisfied no creatine precursors are left over to be metabolized into creatine.

Let us take as our first example for the discussion of the effect of protein feeding on creatinuria, the case of the actively growing animal. For growth, an animal has need in the protein of its food of a certain amount of those amino-acids which it cannot synthesize. An example of such an amino-acid we have in

[26] Shaffer, P. A., and Coleman, W., *Arch. Int. Med.*, 1909, iv, 538.
[27] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 305.

lysine. If an ingested protein is primarily poor in lysine and unable to cover the requirements of the growing animal for it, then the other amino-acids, even if they are present in proper amounts and proportions to make the new protein molecule, cannot all be retained. They are retained only to the extent that the growth determinant lysine makes this possible. The excess is metabolized to obtain its energy and the nitrogen is excreted as waste in the urine. Some of this nitrogen appears as urea and some of it, if creatine precursors are present in excess relative to the demands for protein synthesis, as creatine. The inevitable result of such supplementary relations would be, that a fortunate selection of kind as well as amount of protein would lead to creatine production in one case and to a suspension of its formation in another. This would depend on whether in the supplementary relations all or only part of the creatine precursors in the system could be used up in the synthesis of the new protein molecule. Any factor influencing growth would affect the creatine excretion.

In periods of excessive endogenous protein metabolism leading to the elimination of creatine, the action of feeding protein with respect to its effect on the creatinuria is entirely similar. Mendel and Rose[8] with fasting rabbits found that creatine elimination was not prevented by feeding fat and protein, but Osterberg and Wolf,[28] Benedict and Osterberg,[24] and later Rose[29] found that in the dog creatinuria induced by fasting was inhibited by protein feeding. Rose and coworkers[30] also made the same observation during fasting in man, but Cathcart,[9] who first studied this problem, did not find it so when giving protein and fat. The Cornell investigators, as well as Rose, were inclined to attribute the disappearance of creatine to the action of the glucose which could have its origin in the protein molecule. The administration of an equivalent amount of glucose was observed to have the same effect and moreover in diabetes with failure of ability to utilize the carbohydrate which might have its origin in the protein molecule, protein feeding was ineffective. It may well

[28] Osterberg, E., and Wolf, C. G. L., *J. Biol. Chem.*, 1908, iv, p. xxiii.
[29] Rose, W. C., *J. Biol. Chem.*, 1916, xxvi, 331.
[30] Rose, W. C., Dimmitt, F. W., and Cheatham, P. N *J. Biol. Chem.*, 916, xxvi, 339.

be that part of the action of the protein can be attributed to this factor, but we believe that one more important is to be found in the supplementary relations of the amino-acids ingested to those liberated in endogenous protein catabolism. It is now demonstrated that for repair processes during maintenance the amino-acid demands are different from those during growth. This must mean as has been expressed before from this laboratory[31] that in endogenous protein catabolism the destruction of all amino-acids is not in proportion to the extent in which the individual amino-acids occur in the disintegrating protein molecule. As the body protein is catabolized creatine precursors are liberated and creatine appears in the urine. On feeding protein the ingestion of sufficient amounts of those amino-acids already destroyed makes possible the retention of the creatine precursors in protein synthesis and creatine disappears from the urine. If, however, the ingested protein is unusually rich in creatine precursors then creatine does not disappear from the urine as excessive amounts of it will now be formed from the exogenous protein metabolism.

In the lactating animal we have again the same though slightly modified conditions. Mellanby[32] observed that: "The increase in weight of healthy children breast fed under similar normal conditions is roughly proportional to the amount of creatine in the mother's urine. In other words, the creatine excreted in the urine seems to have some relation to the nutriment given by the mother to her child." Apparently we have here another instance where the relation of creatine production to protein catabolism is evident. We may accept that the creatine here observed has its origin in the unusually large amounts of protein consumed by the lactating mother, of which the excessive amounts of creatine precursors are metabolized to creatine, but creatinuria in this case might also owe its origin to the disproportionate amounts of creatine precursors ingested to the sum total of the requirements imposed by body protein conservation and milk protein production.

In the foregoing, while we have often spoken of both creatine production and creatine excretion we have held in abeyance a

[31] McCollum, E. V., *Wisconsin Exp. Station, Research Bull. 21*, 1912.
[32] Mellanby, E., *Proc. Roy. Soc., Series B*, 1913, lxxxvi, 88.

discussion of the discrepancies which may obtain between the amounts of creatine produced and the amounts of creatine excreted. Until, as Folin and Denis[33] have emphasized, more data are available on the variation in the creatine content of body tissues, it is not possible to discuss this phase of the problem at length, but we do believe that many of the interpretations of data on creatine production and elimination are faulty because of failure to take cognizance of the fact that creatine may in part be utilized or destroyed.

That the animal body has a fairly definite ability to destroy creatine and that in creatinuria creatine has been produced in such amounts that the ability of the body to destroy it has been exceeded, is especially suggested by Folin's[19] observation that creatinuria could be induced far more readily by creatine feeding in man when the subject was receiving a high protein diet than when receiving a low protein diet. Under such circumstances while the creatine ingested did not exceed in amount the ability of the subject to destroy it, the sum total of the creatine ingested together with that having its origin in the protein did exceed it and creatinuria resulted.

Apparently, however, Folin or Denis[33] are not inclined to consider creatine a waste product, as they have stated: "Creatine must, however, be a waste product or a synthetic product serving some special function or as a synthetic product it may in fact be a part of the active living protoplasm. We believe that the last named alternative represents the facts, and in support of this hypothesis we now propose to show that the so-called creatine of muscles is a post-mortem product and that there is very little creatine in living muscles." However, their later work, as well as ours, which shows creatine excretion to be induced by liberal protein feeding, would hardly support the statement that creatine at least under certain entirely normal conditions is not a waste product. Benedict and Osterberg[24] have stated: "Creatine is probably being formed in the animal organism in relatively large amounts, and is normally for the most part either utilized or destroyed." Rose and Dimmitt[34] state: "We believe that these data indicate that the theory accepted without direct proof

[33] Folin and Denis, *J. Biol. Chem.*, 1914, xvii, 493.

[34] Rose, W. C., and Dimmitt, F. W., *J. Biol. Chem.*, 1916, xxvi, 345.

by so many investigators, namely, that creatine and creatinine are destroyed in the body, is without foundation in fact."

The difficulty in accepting the hypothesis of creatine destruction lies in the fact that most of the nitrogen of the ingested creatine does not appear in the urine, as Folin[19] observed in the first experiments which led him to consider creatine as a food. Nor have Folin[19] or others[34] found evidence to warrant accepting its transformation into urea or ammonia. There is evidence that in part it may be changed to creatinine, but the larger portion is excreted as unchanged creatine. If it were destroyed we should expect that the urinary constituents, *viz.* ammonia or urea, would be increased, or at any rate that the urinary nitrogen after subtracting creatine and the increased creatinine nitrogen would still show an increase. We can come to only one conclusion and that is that in such instances the creatine was being stored.

That muscle tissue may be exceptionally low in creatine in physiological as well as pathological conditions has been observed by Howe and Hawk,[6] Denis and Kramer,[16] and Baumann and Hines.[35] Acceptance of the storage possibility does not presuppose that an exceptionally high creatine content of muscle need obtain upon the failure of ingested creatine to make its appearance in the urine. Teleologically a more than incidental or moderate accumulation of creatine during creatine administration in the tissues of the body would lead us to be inclined to attribute a specific function to creatine. So far as we are aware, there is no indication that creatine has any specific rôle to perform in the animal body unless it is that by reversible reaction it can serve as a source of the guanidine grouping for arginine synthesis. Such reasoning would lead us too far into the hypothetical without experimental basis to make it worth more than a suggestion for future work.

During short periods of high level ingestion of creatine or creatine precursors, creatine storage might be assumed to occur as the tissues serving for creatine storage might have been originally depleted in creatine; but during long periods of observation where excessive amounts of protein are being fed with no resulting appearance of creatine or increase in creatine excretion the situation cannot be explained so easily. Especially is this true

[35] Baumann, L., and Hines, H. M., *J. Biol. Chem.*, 1917, xxxi, 549.

of mature men where there can be no extensive utilization of creatine precursors in tissue construction and therefore of necessity we would infer that the conditions were especially propitious for the formation of large amounts of creatine. Folin and Denis[33] have made the statement that 1 gm. of creatine per day is about the maximum which a full grown man can be made to retain even when kept on low protein diets, and on high protein diets the amounts retained are smaller than on diets low in protein. We are forced to the conclusion if we accept the rest of our theory that either the metabolism of creatine precursors in these individuals proceeds primarily in another direction from that leading to creatine (with arginine this would mean breaking up the guanidine residue) or that the destructive ability of the individual for creatine itself is greater.

By our statements here we hope that we have sufficiently brought out the conditions which our theory of the origin of creatine in the urine would demand so that it may be clearly proved or disproved in other laboratories as well as in our own. It may even be that creatine does not have its origin in the protein metabolism of the exogenous character, but that the metabolism of the ingested protein so stimulates the endogenous metabolism that creatine is produced from it in larger amounts and excreted. With the information at present available we are not inclined to champion this latter theory, but hope later to offer data which will make the precursor theory of the origin of creatine entirely acceptable.

In addition, we desire to present a suggestion in regard to the cause for the difference in the behavior of men and women in the facility with which creatinuria is produced on ingestion of protein. We believe it possible that this may be related to the variations well known to obtain in the activity of the thyroid mechanism. To support this, we have the observation of Denis[20] that in a man afflicted with Graves' disease creatine was observed in the urine even when on a low protein diet. Yet in normal well nourished men so far as we are aware, creatinuria has never been observed or experimentally produced. This led to the early statement that adults did not have creatine in the urine. In women, however, creatinuria usually obtains. Krause[36] at-

[36] Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293.

tempted to relate this to the sexual cycle, but M. S. Rose[25] could not establish such a relation. It is suggestive that periodic variations in thyroid activity occur with the menstrual period varying in degree with different women and that during pregnancy when the thyroid is also affected pronounced creatinuria has often been reported. Such data are too limited and have been collected without regard to consideration of the character of diet, so their value is problematic. In this connection the work of Henderson,[22] previously mentioned, is suggestive in that changes in creatine content of muscle have been observed in tetania parathyreopriva. The relations of the endocrinal secretions to creatinuria might well be investigated.

SYNTHETIC CAPACITY OF THE MAMMARY GLAND. I.

CAN THIS GLAND SYNTHESIZE LYSINE?*

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(Received for publication, September 30, 1918.)

How far special glandular tissues of the body have preserved specific synthetic functions not shown by muscle cells and less active tissue cells is a question concerning which we have little definite knowledge. It is recognized that specialized glands are constantly making products unique in function and very specific in constitution, as for example the enzymes, the ductless gland secretions, etc.; but from how simple a precursor,—simple in chemical structure,—this is accomplished is practically unknown.

The mammary gland synthesizes a special sugar, as for example lactose, but it probably starts with *d*-glucose, which as a precursor offers no unsurmountable difficulties. It makes a variety of fatty acids of special chemical character, but here again, whether originating from carbohydrates or preexisting fats, the chemical steps, at least theoretically, are capable of projecture. This gland makes a special protein, casein, and presumably from amino-acid fragments in the blood. It is also pres med that it makes casein, not through synthesis of amino-acids *de novo*, but by an orderly assembling, in quantitative relations, of those amino-acids found in the casein molecule. Should an amino-acid, occurring in casein and essential for growth, be absent in the diet, would there be a serious interference in casein building? Or does this gland retain special synthetic powers not shown by muscle cells, and in the absence of a specific amino-acid make it from other nitrogenous fragments? If, for example lysine, an amino-acid necessary for growth, is absent from the

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diet, will the mammary gland still make casein and continue a copious flow of milk?

In 1912, Osborne and Mendel[1] published experiments on the rôle of gliadin in nutrition in which they found that with gliadin as the sole source of nitrogen the capacity of the animal to produce healthy young and suitably nourish them was not impaired. At that time gliadin was held to be a protein free from lysine and those experiments left the impression that the mammary gland could synthesize lysine. Later work by Osborne, Van Slyke, Leavenworth, and Vinograd[2] showed that gliadin, purified as far as possible, still contained 1.21 per cent of lysine. In fact, in the first paper by Osborne and Mendel referred to above,[1] the question of the freedom of gliadin from lysine was raised and analysis of the gliadin used showed 0.15 per cent of lysine. In addition to the fact that the gliadin used in the above experiments contained lysine, the use of natural, protein-free milk was practised. While the nature of the nitrogen of the latter material has not been definitely characterized, its efficient supplementing effect, at least for certain materials, has been definitely established.[3]

Therefore, in view of the possible errors creeping into these earlier experiments and the rather important fundamental question at issue, namely the synthetic power of the mammary gland, experiments, initiated 2 years ago for further study of this question, were evolved with zein as the basal protein rather than gliadin. Zein was selected because it is a protein recognized today as free from lysine and tryptophane, and the biological value of this protein harmonizes more fully with its chemical deficiencies than does gliadin.

EXPERIMENTAL.

Our plan of procedure involved the use of zein diets made complete by properly supplementing with a complete salt mixture, vitamines, tryptophane, and lysine as contrasted with the

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 473.

[2] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

[3] Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxi, 149.

same diet free from lysine. In some records, because of the relative poverty of lysine in arginine and histidine, both of these amino-acids were added in order that the essential amino-acid content of the ration should be approximately that of casein. The source of water-soluble vitamine in some of our rations was an alcoholic extract of milk powder, while in other rations brewers' yeast was used directly. Our earlier experiments indicated that commercial milk powder was not an abundant source of the water-soluble vitamine. Either this vitamine exists in such stable combination as to resist solution in 95 per cent alcohol, or it has been partly destroyed in the milk-desiccating process. It is also certain that the extraction made with hot 95 per cent alcohol slowly destroys this vitamine.

Tryptophane was prepared in the usual way by pancreatic digestion of casein, and lysine, arginine, and histidine were obtained by acid hydrolysis of casein. Lysine was introduced into the ration as the hydrochloride, while arginine and histidine were introduced as carbonates. Special care was used in the preparation of zein from gluten meal. It was purified by re-solution in alcohol a number of times (three) and finally evaporated on dextrin from an alcoholic solution.

The qualitative composition of the ration was as follows, although somewhat varied in succeeding experiments (see charts). The salt mixture used was the one found in this laboratory as suitable for synthetic rations.

Composition of Ration.

	per cent
Zein	19.46
Tryptophane	0.54
Lactose	20.00
Dextrin	48.00
Butter fat	5.00
Agar	2.00
Salts	5.00

Composition of Salts.

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Iron citrate	0.118
Ca lactate	1.300
KI	In traces.

In our earlier experiments the dextrin carried an 8 hour, 95 per cent alcoholic extract of 480 gm. of milk powder per kilo of ration as the source of water-soluble vitamine, but as we became suspicious of its inefficiency as a source of this vitamine, dried

brewers' yeast (0.5 to 2 per cent of the ration) was used as the source of this dietary factor. Preliminary experiments showed that this yeast must be introduced in the proportion of at least 2 per cent of the ration. Less than that amount was insufficient for normal performance.

When lysine was introduced it formed 1 per cent of the ration. In the case of arginine and histidine each was added as 0.5 per cent of the ration.

Vigorous female rats were selected from our rat colony as experimental animals. It was planned to have an animal on the ration some time before it was bred, in order that the effects of the ration would not be obscured by reserve food materials in the digestive tract. In experiments of this character there are always dangers of incorrect interpretation, due to the possible use of reserve tissues for the more special functions of important organs; while this source of error cannot be entirely avoided there should at least be preliminary feeding periods of sufficient length to secure complete replacement of the old diet by the new. However, as our work progressed we were impressed with the great difficulty in securing pregnant animals on lysine-free rations and for that reason preliminary feeding periods were often short. This did not appear to vitiate the results, as those animals on diets free from lysine were invariably unable to rear their young, while a pronounced improvement in the growth and duration of life, although not a perfect rearing, was secured only when lysine was introduced into the diet. We submit these records as evidence that the mammary gland has not the power to synthesize the amino-acid lysine, but must find it preformed in the blood stream.

It appeared possible for the mother rat to build her young, presumably from tissue reserves rather than through synthesis, even in the absence of lysine from the ration. We had a number of animals give birth to apparently normal litters of young (see Charts 1 to 5) on rations free from lysine, but only in a single case was a litter nursed sufficiently to carry it beyond a mere maintenance of life for approximately 2 weeks after birth. In one case part of the litter was nursed for $3\frac{1}{2}$ weeks and then all succumbed. It is apparent from our results that the amount of nitrogen furnished by 2 per cent of yeast in the ration was too poor in quality and too low in quantity to supplement our lysine-

free ration sufficiently for normal milk flow. We are decidedly of the opinion that what milk was secreted was normal rat's milk and not a milk whose proteins were deficient in lysine. It was in all probability a diminished flow, but a milk of normal composition.

This principle, namely that the composition of the milk produced under distinctly adverse nutritional conditions will be approximately normal in composition, is firmly established wherever it has been possible to apply quantitative analytical methods, as for example in the case of cow's and goat's milk. True, slight variations in milk composition may occur with variations in the diet, but not sufficient to disturb the *nutritive* qualities of the milk.

The work of Palmer and Eckles[4] has definitely established the fact that the mass of the main coloring matter in milk, carotin, is directly dependent upon its supply in the feed ingested. Further, of great interest were the experiments of Andrews[5] in which Philippine women, suffering from beri-beri and who had lost their infant babies, were induced to nurse young pups. These pups developed paralysis of the posterior extremities and exhibited the symptoms characteristic of beri-beri. Because of the fact that this disease is directly related to the deficiency of the water-soluble vitamine in the diet, these experiements were interpreted as indicating that the milk secreted by these women was likewise deficient in this essential.

On this point these experiments, however, are not absolutely conclusive. The rate of growth of the human baby is quite different from that of a pup. Human milk, whenever secreted, may contain the water-soluble vitamine in sufficient quantity to meet the rate of growth of the human infant, but not of a pup. The protein content of human milk is much lower than that of the milk secreted by the bitch, and we should not expect that the former would meet the protein requirements of growing pups. Why should we ignore in experimental inquiries of this kind the firmly established principle of the adaptability of the milk of a species to the young of that species? Failure of cow's milk to meet the complete nutrition of a rat must not be interpreted as a

[4] Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191.

[5] Andrews, V. L., *Philippine J. Sc., B.*, 1912, vii, 67.

deficient milk for a calf or for a human infant. The rat doubles its weight in 14 days, while it requires 47 days for the calf to double its weight, and 180 days in the case of the human infant.

In harmony with the idea of a vitamine deficiency and conforming to the principle of species adaptability mentioned above, Andrews is of the opinion that the high infant mortality in Manila is due to the deficiency of water-soluble vitamine in the mother's milk. However, no records of flow of milk accompany his discussion. In addition to Andrews' work, that in this laboratory of McCollum[6] and of Steenbock[7] with barley supports the view that both the fat- and water-soluble vitamines of milk are dependent upon their supply from the food and cannot be synthesized by the mammary gland. Therefore, with the exception of coloring matter and probably with the exception of vitamines, although at the present time we are lacking absolutely decisive data on this point, the other organic and inorganic constituents of milk remain practically constant under wide variations of diet.

When the milking animal, through scarcity of structural units, is forced to conserve, it lowers the flow of milk, but maintains the normal composition of the milk. These facts are so well known to investigators of dairy problems that reference to but a single text[8] and to Babcock's work[9] on withholding chlorides from the rations of cows, is necessary to support this view. In Babcock's experiments common salt was withheld from the rations of dairy cows for 5 to 6 months without an appreciable change in the chlorine content of the milk. It contained 0.10 per cent of chlorine at the initiation of the experiment and 0.08 to 0.09 per cent when the critical period was reached. The critical period was that point at which a sharp decline in milk flow set in, with other manifestations of malnutrition. As soon as the chlorides were readministered the tendency was toward a normal flow.

[6] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33.

[7] Steenbock, H., Kent, H., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxv, 61.

[8] Jordan, W. H., Feeding of farm animals, New York, 1917.

[9] Babcock, S. M., *Wisconsin Exp. Station, 22nd Ann. Rep.*, 1905.

These facts, however, must not be confused with the variations in individuals to produce small or large flows of milk on the same rations; nor with the probable ability of cows, with pronounced dairy breeding, to maintain unusual flows of milk of normal composition although receiving wholly inadequate rations as compared with mother rats, where the mammary function has been developed only to supply milk sufficient for the normal growth of the litter.

Where lysine was added to the zein-tryptophane ration not only were normal litters of young born, but in most instances the young were successfully nursed. In no case was the growth of the young at a normal rate, although the litters were reduced to either four or five individuals. In one record (Chart 6) where the source of the water-soluble vitamine was a 95 per cent alcoholic extract of Merrell-Soule skimmed milk powder the behavior of the young indicated a deficiency of this essential in the milk secreted. At the age of 3 weeks they began to be seized with convulsions, loss of muscular control, and thereafter gained little in weight; death terminated their life at the end of 5 weeks.

In other records on the zein-tryptophane-lysine diet, but with the water-soluble vitamine supplied through the introduction of 2 per cent of yeast, no such symptoms of vitamine deficiency developed in the young (see Charts 7 and 8). There was not a normal rate of growth of these young, but a steady, slow growth and an apparently healthy condition of the mother rat and young. The introduction of the lysine had improved the ration to such an extent as to make continued milk secretion possible, while without it the animal was limited merely to the building of the young and an exceedingly scanty flow of milk.

The probable cause of failure to rear the young at a normal rate on a zein-tryptophane-lysine diet was the incomplete absorption of the zein itself. It is not probable that our supply of lysine was inadequate as it constituted 1 per cent of the ration, a quantity five times as large as used by Osborne and Mendel[10] in successful growth experiments with zein. Osborne and Mendel[11] have published data showing the incomplete utilization of

[10] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxv, 1.
[11] Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 177.

zein by rats. While our ration contained approximately 20 per cent of zein, yet if its utilization was reduced 30 to 50 per cent by failure to be absorbed, as in the case of the experiments quoted above, it can readily be understood how milk flow would be greatly reduced. In support of the view that our purified zein ration was partly unsuccessful for rearing the young because of the indigestibility of the zein, we submit a record (Chart 9) showing the successful rearing of the young at normal rates by substituting casein for the zein-tryptophane-lysine combination in the diet. With the same percentage of nitrogen in the ration the casein was much more effective in stimulating and maintaining milk secretion than was the zein-tryptophane-lysine complex. This we attribute to a more complete absorption of the protein, casein.

It was also possible that our zein-lysine ration was limiting milk flow by its paucity in arginine and histidine. These amino-acids are not as abundant in zein as in casein. For that reason there was added to the zein-tryptophane-lysine diet at the rate of 10 gm. per kilo, a mixture of arginine and histidine obtained by the usual separation of these amino-acids from an acid hydrolysis of casein.

The results secured with this ration are shown in Charts 11 and 12. Normal sized litters of young were born and then reduced to four in number. These were nursed by the mother rat and made slow rates of growth considerably below a normal performance. The rate of growth of these young was no greater than on the ration in which the arginine and histidine additions were omitted. Apparently the indigestibility of the zein was counteracting any beneficial effect the arginine and histidine might have had when added to a protein relatively low in these amino-acids but more available.

To satisfy ourselves that the 2 per cent of yeast introduced into the ration would meet the water-soluble vitamine requirement of growing rats a ration was made up with casein as the sole protein, while another ration contained the zein-tryptophane-lysine complex as the carrier of nitrogen. The rats were started at initial weights of 40 to 70 gm. with casein as the sole protein (see Chart 10). Normal growth was secured with 2 per cent of yeast furnishing the water-soluble vitamine. The yeast

used in all our experiments was a dried powder, secured from a Chicago Brewing Company. It was reported to us that in its preparation it had been exposed for a few seconds to a temperature of 176°C.

Since the casein-yeast ration was entirely satisfactory for growth, any deficiency in the zein-tryptophane-lysine ration could, with justice, be assigned to the nitrogen complexes. This deficiency is probably to be attributed mainly to the low digestibility of the zein. Chart 13 shows the growth curve of rats upon the zein-lysine ration containing 2 per cent of yeast. These growth curves were below normal and demonstrate that our ration was still below the possible attainment in synthetic diets containing a more complete and more digestible protein such as casein.

A review of the records secured with our lysine-free ration indicates that in the majority of cases these animals do not even maintain their live weights. Other investigators[12] have published results which indicate that either lysine was dispensable for maintenance or at least not needed to the same degree as, for example, tryptophane. The fact that there is a rapid decline in body weight in the absence of tryptophane, but only a slight decline, or in some cases actual live weight maintenance for periods of many weeks' duration in the absence of lysine, forces one to the conclusion that the above interpretation of their records by Osborne and Mendel and by Geiling was justified. However, in our own records (see Charts 1 to 3) with lysine-free diets there was a steady decline in live weight in two cases, but a phenomenal maintenance of weight in one case (Chart 1) over a period of 24 weeks. These records raise the question, was there really normal maintenance and normal functioning in these animals? We think not. Their coats became rough and soiled, which is always evidence of malnutrition. These rats were extremely nervous, excitable, and old ahead of their time. Estrum periods disappeared, which certainly is not compatible with normal maintenance.

[12] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325; 1916, xxv, 1. Geiling, E. M. K., *ibid.*, 1917, xxxi, 173.

Further, in view of the fact that maintenance of live weight is not a sound basis[13] upon which to decide whether or not an animal is in nitrogen equilibrium, we are forced to the conclusion that *normal* maintenance is not possible in the absence of the amino-acid, lysine. In such maintenance records there certainly is the possibility that catabolyzing non-essential tissues can furnish over very long periods of time enough lysine for rehabilitation of those tissues more essential for the maintenance of life. The maintenance of body weight may be either a replacement of loss with water or with fat.

SUMMARY.

The evidence presented in this paper makes it very probable that the mammary gland has not the capacity to synthesize the amino-acid, lysine.

Further, the evidence submitted supports the view that as far as the proteins are concerned milk secretion, like growth, is ultimately dependent upon the quality and quantity of amino-acids ingested with the food.

Our records also lead us to the view that the amino-acid, lysine, is not dispensable for *normal* maintenance.

[13] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1918, xxxv, 367.

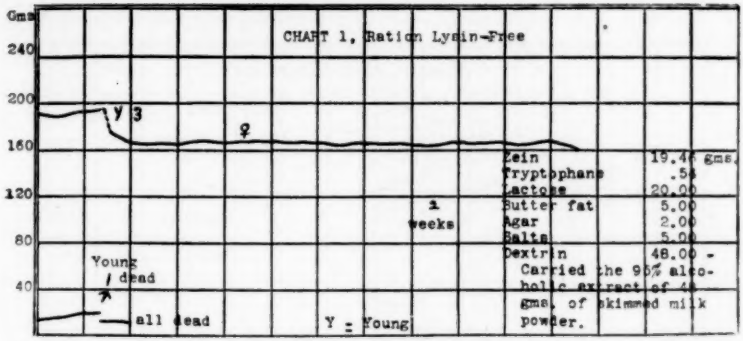

CHART 1. On a lysine-free ration young were born, but not successfully nursed. We believe that this is evidence that the mammary gland has not the power to synthesize lysine. Sacrifice of tissue at a rate sufficient to furnish an adequate supply of milk for milk protein building was not possible. On the other hand, we must in these cases assume that the lysine needed for fetus building came from the catabolized tissue of the mother rat. This rat maintained its live weight on the lysine-free diet, but was rough in appearance and showed no signs of estrum. We doubt if continuous, normal maintenance is possible on a lysine-free diet.

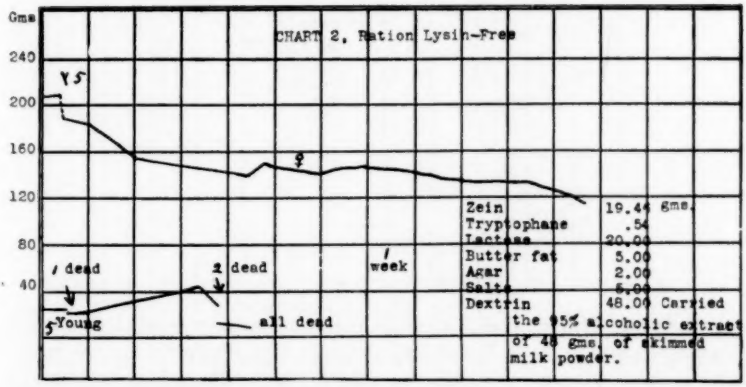

CHART 2. Another failure to rear the young on a lysine-free diet. Milk secretion was probably more abundant in this case than in the case of the record shown in Chart 1, but the rapid loss in live weight would indicate a more rapid tissue metabolism under a stronger impulse for mammary secretion. Further, the shortness of the interval between the time of being placed on the ration and the birth of the young made possible a larger store of lysine-yielding tissue for milk secretion.

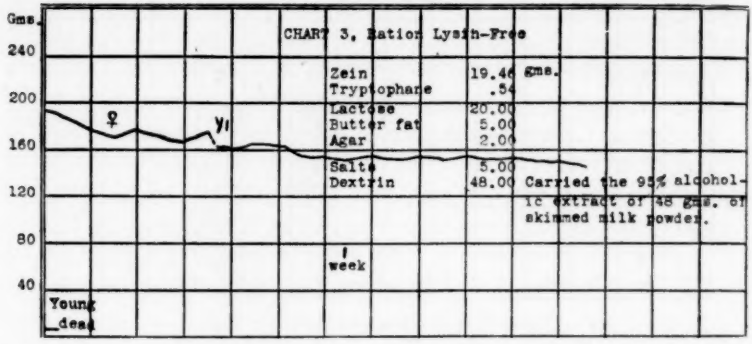

CHART 3. No rearing of the young on a lysine-free ration, but practically a maintenance of live weight after the young were born. Here again the mother rat was rough in coat and far from normal in appearance. We interpret these results as indicating that normal maintenance is impossible without lysine.

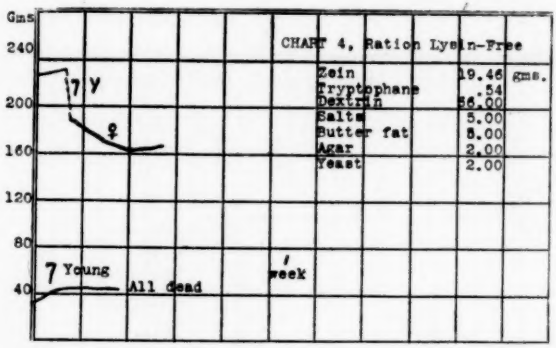

CHART 4. This mother rat, as in previous cases, was wholly unable to nurse her young on a lysine-free ration. The young lived less than 2 weeks, procuring a little milk for maintenance; the lysine, contained in the milk proteins made, undoubtedly was furnished through tissue catabolism by the mother. Even the presence of 2 per cent of yeast in the diet did not furnish nitrogen in quality and quantity sufficient to supplement adequately a zein-tryptophane ration for milk production.

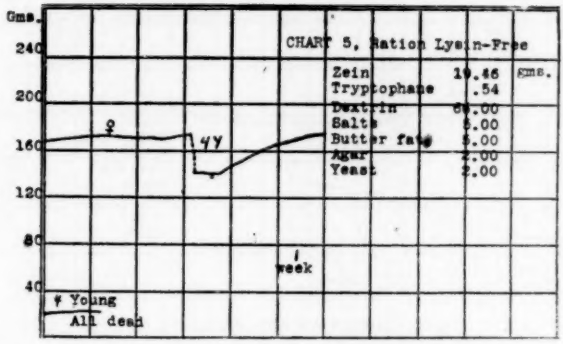

CHART 5. Another failure to rear the young on a lysine-free ration, but containing 2 per cent of yeast as the source of water-soluble vitamine.

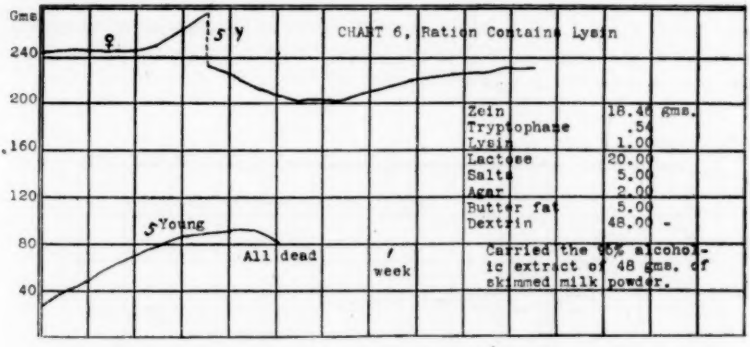

CHART 6. The beneficial effect on milk secretion of lysine in the ration. In the presence of this amino-acid the young were nursed for nearly 5 weeks, at the end of which time there was a sharp decline in weight, and death. 1 kilo of the ration contained the alcoholic extract of 480 gm. of skimmed milk powder. The behavior of the young, characterized by great excitability and impaired locomotion, would indicate a deficiency of the water-soluble vitamine in this ration. That this interpretation of our results is correct is evidenced by the data shown in Charts 7 and 8 where 2 per cent of yeast in the ration was used.

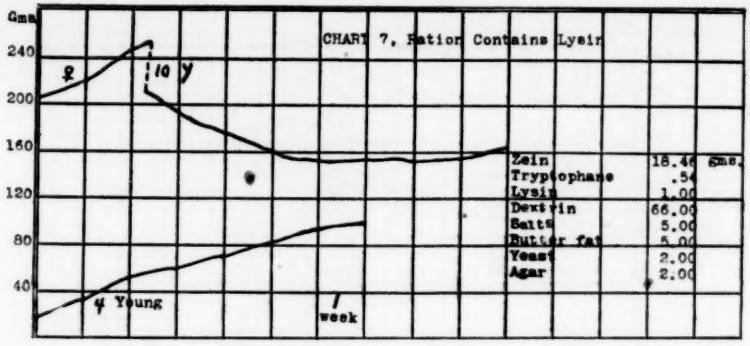

CHART 7. This chart illustrates the effect of both lysine and an adequate supply of water-soluble vitamine furnished by 2 per cent of yeast. This mother rat gave birth to a litter of ten young. We reduced the litter to four in number which were nursed with fair success. Normal rates of growth of the young were not obtained, due no doubt to a diminished milk flow occasioned by the low digestibility of zein. The young, however, were strong and active, but below normal size. For comparison see Charts 4 and 5, which demonstrate that the improvement in the ration was due to the lysine and not to the yeast. With lysine in the ration more copious milk secretion was made possible.

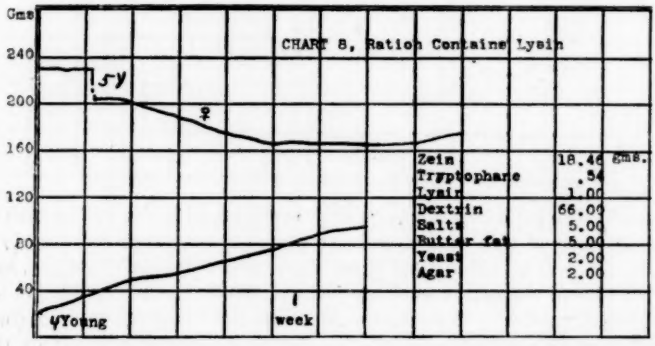

CHART 8. A duplicate of Chart 7 showing the marked effect of lysine in the diet on milk secretion. Without lysine there would have been no rearing of the young. The young grew, however, at a rate below normal, due to the insufficient flow of milk, but were nursed and finally weaned.

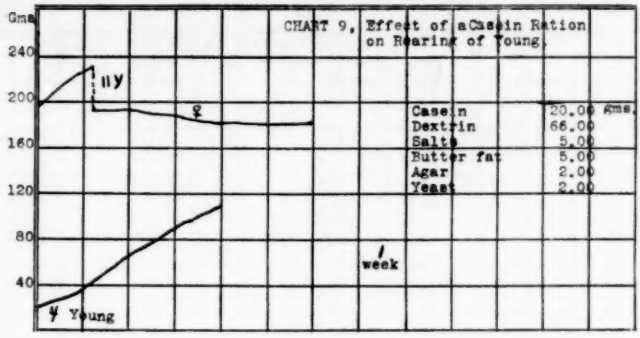

CHART 9. The effect of a good protein on milk secretion. In this ration casein in quantitative equivalence was substituted for the zein-lysine-tryptophane mixture; otherwise the rations were identical. The litter of eleven young was reduced to four in number. The young grew at a much more rapid rate than the litters shown in Charts 7 and 8. With higher efficiency the more completely absorbed casein met the needs of maintenance and a more copious milk secretion than did the less readily digestible protein, zein. This chart further indicates that 2 per cent of yeast could furnish the water-soluble vitamine in quantities sufficient for a nearly perfect rearing of at least four young rats.

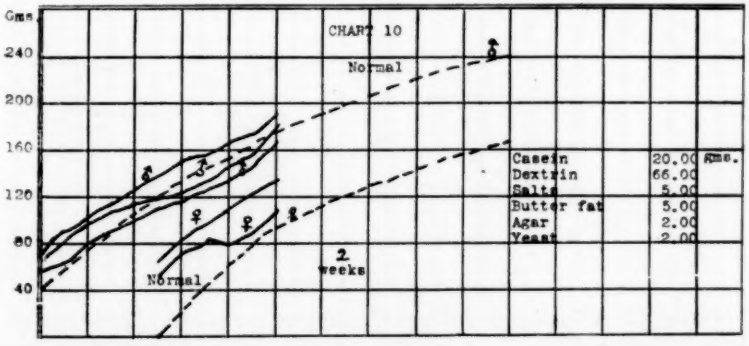

CHART 10. This chart shows that the yeast used, constituting 2 per cent of the ration, was able to furnish sufficient water-soluble vitamine for normal growth of young rats where the other factors of nutrition were adequately provided. These animals were in excellent condition.

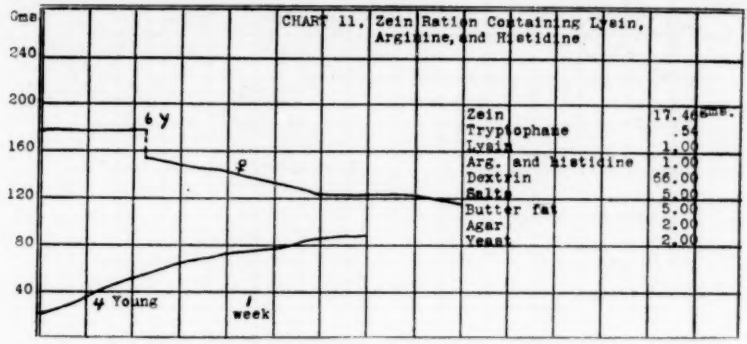

CHART 11. This chart illustrates the effect on milk secretion of adding arginine and histidine to a zein-lysine-tryptophane ration. The addition did not improve the ration for milk secretion. The young made no better growth than those whose mother received a zein-tryptophane diet fortified only with lysine. The indigestibility of the zein was the principal factor depressing the efficiency of this ration for milk secretion.

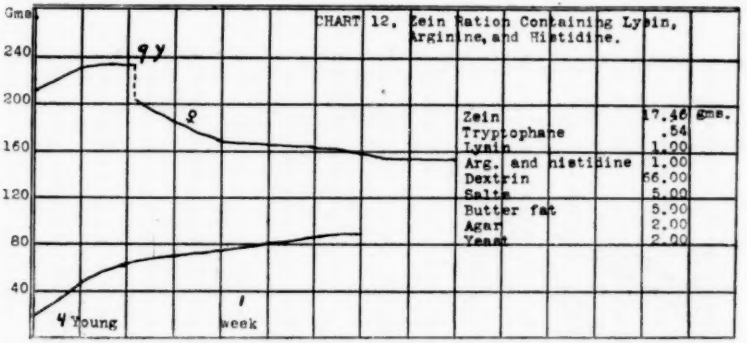

CHART 12. A duplicate of Chart 11, showing the failure of additions of arginine and histidine to a zein-lysine-tryptophane ration to make this nitrogen complex equal in nutritive value to that of an equivalent amount of casein. While the young were nursed and reared to the time that they could be weaned, yet their rate of growth was not a normal one.

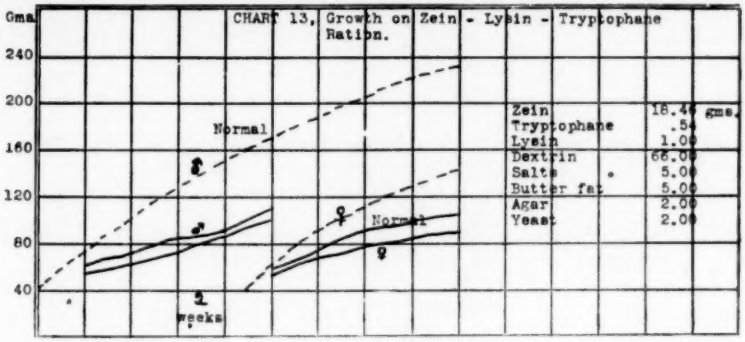

CHART 13. The curves of growth of young rats on the zein-lysine-tryptophane ration. Started at 50 to 60 gm. in weight, nevertheless they were unable to make normal growth on this ration. Compare with Chart 10 where casein, as the sole protein and supplemented in reference to the other nutritive factors in identically the same manner as was the zein-lysine complex, served to support normal growth.

COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS. II.

FATE OF *P*-HYDROXYLBENZOIC ACID AND *P*-HYDROXYLPHENYLACETIC ACID IN THE ORGANISM OF THE MONKEY.

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It has previously been shown that phenylacetic acid in the organism of the monkey undergoes the same process of metabolism as in the lower animals, and is subsequently excreted in combination with glycocoll as phenaceturic acid (1). On the other hand, if ingested by man it undergoes apparently an entirely different process of metabolism, being excreted in the urine in combination with the amino-acid glutamine (2) and known as phenylacetyl glutamine. From a biological standpoint it is of interest to know how far the metabolism of the monkey belongs to that of the lower animals or to that of man in those points where a difference has been demonstrated. Other investigators have shown that the monkey follows the course of the lower animals in the purine metabolism (3) as well as in the ratio of ethereal sulfates to inorganic sulfates (4) excreted in the urine. These isolated facts have little or no value until we have secured enough data to correlate and in this way to follow the fate of each amino-acid during the entire process of metabolism. *p*-Hydroxylbenzoic acid and *p*-hydroxylphenylacetic acid are two members of a series of compounds which result from the action of putrefactive bacteria on the aromatic amino-acids. These compounds, all of which are more or less toxic, are quickly detoxicated and rendered sufficiently soluble for their elimination in the urine. The method of detoxication is usually by conjugation with the sulfate radical, glycuronic acid, one of the shorter amino-acids, such as glycocoll, cystine, or glutamine, or

even at times with urea. Authors do not entirely agree as to the fate of the aromatic hydroxyl acids or as to the method of their elimination. One fact seems to be universally accepted, that *p*-hydroxylbenzoic and *p*-hydroxylphenylacetic acids are among the least toxic of all the putrefactive products thus formed.

The former acid when fed to dogs was found in the urine as such by E. Salkowski and H. Salkowski (5) with an augmentation of the quantity of ethereal sulfates, indicating as they believed, a conjugation with the sulfate radical. Baumann and Herter (6) found a decided increase in ethereal sulfates after administering a dose of 2 gm. to a dog but failed to find this same condition after administering an equivalent amount to a human being. They recovered most of the acid from the dog's urine unchanged but found also a trace of *o*-hydroxylhippuric acid. Bertagnini (7) claims to have found relatively large amounts of *o*-hydroxylhippuric acid after animal feeding with the *o*-hydroxylbenzoic acid. Maly (8) after ingesting the acid himself, finds a compound in the urine which he believed to be a conjugation of *p*-hydroxylbenzoic with ethyl or methyl glycocoll, but this, as Schotten (9) explained, was on account of error in analysis, and the compound recovered from the urine was undoubtedly *p*-hydroxylhippuric acid. Schotten ingested 26 gm. of *p*-hydroxylbenzoic acid in the course of 28 hours. He recovered 13.432 gm. (51.66 per cent) from the urine. Of this 9.182 gm. (35.32 per cent) occurred as uncombined *p*-hydroxylbenzoic acid, while 6.004 gm. of the combination with glycocoll was excreted as *p*-hydroxylhippuric acid, which contained 4.25 gm. of *p*-hydroxylbenzoic acid (16.34 per cent). *p*-Hydroxylphenylacetic acid occurs normally in both human urine and in the urine of animals in small amounts. Baumann (10) evaporated 25 liters of normal human urine and isolated from this approximately 0.5 gm. of hydroxylaromatic acids, consisting mostly of *p*-hydroxylphenylpropionic and *p*-hydroxylphenylacetic acids. Again (11), from 240 liters of human urine he isolated 4 gm. of impure *p*-hydroxylphenylacetic acid and a small amount of *p*-hydroxylphenylpropionic acid. These acids he believed to be excreted mostly as the sodium salts and perhaps partly as ethereal sulfates which might have been destroyed in the process of their isolation. Salkowski and Salkowski (12) after feeding 2.5 gm. of *p*-hydroxylphenylacetic acid to a dog, found most of the acid unchanged in the urine but found also a small amount of a substance which came out of solution as "krystallwarzen" (wart-like crystals) in rather an impure condition and melted at 153°. The amount was too small for an analysis but they hydrolyzed a small portion of it by boiling with HCl and were able to isolate *p*-hydroxylphenylacetic acid from the acid solution. They concluded their compound was *p*-hydroxylphenaceturic acid. They do not mention the exact amount of the acid which they isolated from the urine. A repetition of this experiment failed to yield even a trace of the acid. The same dog which had excreted a small

amount of the acid after a 2.5 gm. dose of the *p*-hydroxylphenylacetic acid failed to conjugate any of this acid after a 6 gm. dose and excreted the *p*-hydroxylphenylacetic acid unchanged in the urine. Experiments on other dogs and even on rabbits also met with failure. A rabbit received 2 gm. of *p*-hydroxylphenylacetic acid and excreted 1.25 gm. of the acid in the urine. Schotten (9) ingested 7.5 gm. of the acid and noticed that the urine collected only a short time after gave a strong coloration with Millon's reagent. This reaction continued with decreasing intensity until 10 hours after the ingestion of the acid. From the urine he regained 5.90 gm. (78.6 per cent) of the acid unchanged but found no trace of *p*-hydroxylphenaceturic acid.

In this laboratory a monkey was fed *p*-hydroxylbenzoic acid and *p*-hydroxylphenylacetic acid.[1] The *p*-hydroxylbenzoic acid was in each case excreted in the urine and no combination with glycocoll could be found. The amounts regained were 50, 56.03, and 60.07 per cent of 1, 2, and 3 gm. doses, respectively. Most of the *p*-hydroxylphenylacetic acid also was excreted in the urine unchanged but in one instance 0.78 gm. of *p*-hydroxylphenaceturic acid was found in the urine after a feeding of 2 gm. of the acid, followed 24 hours after by a 2.5 gm. dose. The *p*-hydroxylphenaceturic acid was purified, analyzed, and studied.

EXPERIMENTAL.

A female monkey (*Macacus rhesus*) of 4.2 kilos body weight, was placed on a regular diet of milk, bread, bananas, and apples for several days to determine as nearly as possible the amount of aromatic hydroxyl acids in the urine. The amount of these acids was found to be negligible. The monkey was placed in a small metabolism cage and the urine collected for a period of 36 hours after administering the acid. The acid was given in different sized doses but the entire quantity was given at one time and not throughout a period of several hours. In amounts not exceeding 1 gm. the acid was given as a solution of the sodium salt in milk. In larger quantities a water solution of the sodium salt was introduced directly into the stomach by means of a stomach tube. The different portions of urine excreted during the first 36 hours after the acid feeding were united, carefully

[1] The *p*-hydroxylphenylacetic acid used for this work was prepared by Dr. W. A. Jacobs of The Rockefeller Institute.

neutralized with sodium carbonate, and slowly evaporated on a water bath at low temperature. In most cases the urine after reaching a thick syrupy consistency was cooled, acidified with phosphoric acid until it gave a distinct reaction with Congo red, and then extracted several times for a period of 2 or 3 hours with ether, alcohol, and ethyl acetate in a continuous extracting apparatus. By this means it was possible to make a rough separation between the *p*-hydroxylbenzoic acid and the *p*-hydroxylhippuric in case the latter should be present, and again between the *p*-hydroxylphenylacetic and the *p*-hydroxylphenaceturic acids. In both cases the free acids are much more soluble in ether than their corresponding glycocoll compounds. After extraction, the extract was evaporated to dryness by stages so there was opportunity for crystals to appear at the different concentrations. The residue left was dissolved in warm water and the water solution concentrated at regular intervals during a period of several days. Crystals which appeared were purified by dissolving in water, boiling with charcoal, and again recrystallizing from water. In each case the mother liquor from crystals or the residue from the extraction (if no crystals appeared) was boiled with a 35 to 40 per cent solution of H_2SO_4, cooled, and extracted with ether. By this method it was possible to tell whether any of the hydroxyl acids in a conjugated form had failed to crystallize out of the water solution.

Experiment I with p-Hydroxylbenzoic Acid.—1 gm. of *p*-hydroxylbenzoic acid was fed to the monkey as a solution of the sodium salt in milk. There were no noticeable physiological effects as loss of appetite, dizziness, signs of nausea, or even increased thirst. This seemed to show that the acid is almost non-toxic. The urine for the first 36 hours after the feeding amounted to 130 cc. The general method of extracting was followed. The evaporated urine was extracted three times with ether. Each period of extraction covered 3 hours and about 300 cc. of ether were used for each extraction. All ether was then removed from the extracting apparatus and the urine was extracted for a period of 3 hours with the same amount of alcohol. Again the urine was extracted twice with ethyl acetate. Each extraction lasted about 3 hours and 300 to 350 cc. of ethyl acetate were used. Each of the three ether extracts was evaporated separately; no crystals appeared, so the residue from each was dissolved in hot water, boiled with charcoal, and filtered. After a few hours standing, crystals appeared in the water solution of the first ether extract but none appeared in the water solution of either the sec-

ond or third extract. The water solutions of the three ether extracts were each boiled with acid, cooled, and extracted with ether but no *p*-hydroxylbenzoic acid could be found except the merest trace which probably had failed to crystallize out of the water solution. The alcoholic and ethyl acetate solutions contained small amounts of hippuric acid and much urea which had been carried over mechanically, but even after boiling the residues with acid no measurable amount of *p*-hydroxylbenzoic acid could be found. The crystals from the first ether extract were *p*-hydroxylbenzoic acid, as is shown below. The substance was twice recrystallized from water and dried at 100°; melting point 208–210.°

Analysis.

0.1837 gm. gave 0.4094 gm. CO_2, 0.0729 gm. H_2O.

	Calculated for $C_7H_6O_3$:	Found:
C	60.86	60.79
H	4.40	4.44

Experiment II with p-Hydroxylbenzoic Acid.—After a few days the monkey was given a dose of 2 gm. of *p*-hydroxylbenzoic acid, followed in a few days by a dose of 3 gm. The water solution of the residue from the ether extracts yielded in the first case 1.127 gm. of the free *p*-hydroxylbenzoic acid, and in the latter case 1.822 gm. The mother liquor from these crystals yielded no *p*-hydroxylhippuric acid nor was more than a trace of *p*-hydroxylbenzoic acid extracted with ether after the boiling of this liquid with 40 per cent H_2SO_4. The alcohol and ethyl acetate extracts of these urines yielded only small amounts of *p*-hydroxylbenzoic acid.

Discussion of Experiments with p-Hydroxylbenzoic Acid.

The results obtained from the feeding of this acid are summed up in Table I, which shows that from 50 to 60 per cent of the acid

TABLE I.

	Amount fed.	Amount extracted from urine.	
	gm.	*gm.*	*per cent*
p-Hydroxylbenzoic acid	1	0.5	50
" "	2	1.127	56.03
" "	3	1.802	60.01

fed was recovered from the urine in a free state with the formation of no perceptible amount of *p*-hydroxylhippuric acid. The acid is probably excreted as the sodium salt and perhaps in very small amounts as ethereal sulfate. Baumann and Herter

(6) believed that the sulfate compound existed in the urine but that it was destroyed during the experimental work. This compound of the acid, later prepared synthetically by Baumann (13), proved to be very stable and quite insoluble in organic solvents. It is possible that small amounts of the acid might have been present in the urine as a glycuronic acid compound analogous to that found by Magnus-Levy (14) in the urine of sheep after feeding benzoic acid. The amount of this compound present must have been small, as the optical rotation of the urine before and after the feeding was practically the same.

p-Hydroxylphenylacetic Acid.

The monkey was able to stand as much as 3 gm. of the *p*-hydroxylbenzoic acid (0.685 gm. per kilo of body weight) without any apparent signs of discomfort, but 2.5 gm. of *p*-hydroxylphenylacetic acid seemed to be the maximum dose which the animal could comfortably tolerate. The acid appeared to cause considerable thirst and corresponding higher urine volumes than after *p*-hydroxylbenzoic acid feeding.

Experiment I with p-Hydroxylphenylacetic Acid.—1 gm. of *p*-hydroxylphenylacetic acid was fed to the monkey as a solution of the sodium salt in milk. The urine volume collected the first 36 hours was 186 cc. The urine was evaporated to a small volume, made acid, and was shaken repeatedly with ether in a separatory funnel. About 350 cc. of ether were used for each extraction. The different ether extracts were united and the ether was distilled off. The residue was dissolved in hot water, boiled with charcoal, and filtered. The filtrate was neutralized with Na_2CO_3 and shaken with a large volume of ether to remove pigment matter and other ether-soluble impurities. The water solution was acidified and again extracted with ether. This ether extract, when evaporated left a mass of crystalline material. This material was twice recrystallized from water and dried at 100°. The melting point was between 146.5 and 148° and the substance was therefore relatively pure *p*-hydroxylphenylacetic acid. The urine was next shaken repeatedly in a separatory funnel with large quantities of ethyl acetate. The different portions of ethyl acetate were united and slowly evaporated to dryness. The residue consisted mostly of urea and hippuric acid. The entire mass was dissolved in the smallest possible amount of hot water and left for 24 hours. In this way a few crystals of hippuric acid (melting point. 187°) were removed from the liquid. The entire solution was boiled with an equal volume of 40 per cent H_2SO_4 for 1 hour, cooled, and extracted several times with ether.

As no *p*-hydroxylphenylacetic acid was found in the ether extract it seemed certain that neither the free nor combined acid had previously existed in the ethyl acetate extract of the urine. The entire amount of *p*-hydroxylphenylacetic acid found in the ether extract of the urine was 0.32 gm.

Experiment II with p-Hydroxylphenylacetic Acid.—2 gm. of *p*-hydroxylphenylacetic acid fed to the monkey were followed after 24 hours by a dose of 2.5 gm. The urine was collected for 36 hours after the second dose or for a total period of 60 hours. The entire urine volume was 420 cc., and was strongly acid to litmus. The urine was handled in the usual way but was extracted immediately with ethyl acetate. The evaporated urine for the 60 hour period was extracted three times for periods of 3 hours each with 300 cc. of ethyl acetate in the continuous extracting apparatus. These extracts will be called Nos. 1, 2, and 3. Each day about one-third of the remaining volume of each extract was distilled off and then placed in the ice box during the interval. Quantities of urea crystallized out of each extract but especially out of No. 3. Small amounts of hippuric acid crystallized out of Extracts 1 and 2 as they approached the point of dryness. The residue from Extract 1 was found to be of definite crystalline structure but apparently very impure. The residue from Extract 2 contained the least amount of material, and No. 3 the greatest. These three residues were dissolved in the least possible amount of hot water and neutralized with Na_2CO_3. The water solutions were shaken with ether to remove coloring matter and impurities, then acidified, and again shaken with ether. The last ether extracts were evaporated, the residue was dissolved in water, boiled with charcoal, filtered, and the filtrate allowed to stand in the cold. From the water solutions of Extracts 1 and 2 quantities of *p*-hydroxylphenylacetic acid were regained, amounting in all to 2.177 gm.

The water solutions of the residue from each of the three original ethyl-acetate extracts had thus far yielded only the uncombined acid. In order to recover any of the combined acid still held in the solutions, each water solution of the ethyl acetate residue was shaken many times with ethyl acetate. These ethyl acetate extracts were evaporated and the small amount of material remaining was taken up in hot water. No crystals appeared until the solution was evaporated almost to dryness, when groups of fungus-like masses appeared on the sides and bottom of the crystallizing dish; this was especially so in the case of Extract 1 and to a slight degree in Extract 2. These fungus-like masses were found to consist of groups of minute needles. These groups of crystals were collected and recrystallized from water. When recrystallized from not too concentrated solution they appeared in the form of thin hexagonal plates. In all, 0.78 gm. of this substance was isolated from the urine. The melting point of the substance when dried at 95–100° is 154.5–155°. It is relatively soluble in alcohol, ethyl acetate, and warm water, but quite insoluble in ether, benzene, and cold water. A small amount of the substance was boiled with concentrated HCl for 1 hour, the acid solution was cooled, and extracted several times with ether. The residue from the ether extraction when

recrystallized from water proved by its melting point to be *p*-hydroxylphenylacetic acid. The melting point was 147–148°. When boiled with copper oxide a blue solution of the copper salt results. The copper salt is extremely soluble in water and crystallizes in irregular crystals only when the solution is evaporated nearly to dryness. The compound isolated from the ethyl acetate extract is undoubtedly the glycocoll combination of *p*-hydroxylphenylacetic acid (*p*-hydroxylphenaceturic acid).

Analysis.

a. 0.1021 gm. substance gave 0.2145 gm. carbon dioxide and 0.0488 gm. water.

b. 0.1127 gm. substance gave 0.2370 gm. carbon dioxide and 0.0541 gm. water.

c. 0.1055 gm. substance required 5.05 cc. 0.1 N hydrochloric acid.

	Calculated (Kjeldahl) for $C_{10}H_{11}NO_4$:	Found:	
	per cent	*per cent*	*per cent*
C	57.39	*a*. 57.30	*b*. 57.35
H	5.30	*a*. 5.34	*b*. 5.38
N	6.68	*c*. 6.70	

Discussion of Experiments with p-Hydroxylphenylacetic Acid.

In Experiment I, after feeding 1 gm. of *p*-hydroxylphenylacetic acid, 0.32 gm. (32 per cent) of the acid was recovered from the urine. All of this acid was in the uncombined state and none of the glycocoll combination could be found. In Experiment II, after feeding a total of 4.5 gm., 2.177 gm. were recovered from the urine as the uncombined acid as well as 0.78 gm. of *p*-hydroxylphenaceturic acid. A total of 2.684 gm. of the *p*-hydroxylphenylacetic acid, or 59.64 per cent, was regained after feeding, as compared to 78.66 per cent recovered by Schotten (9) from his own urine after he had ingested 7.5 gm. of the acid. Of this 59.64 per cent isolated from the urine, 48.37 per cent was in the form of uncombined acid and only 11.27 per cent in the form of the glycocoll compound. The failure to find some of the conjugated acid after feeding of the 1 gm. is in contrast to the work of Salkowski and Salkowski (12) who found a small amount of the acid after 2.5 gm. feeding but none at all after feeding the same dog a dose of 6 gm. That the animal organism is able to

furnish much more glycocoll than the amount necessary to combine with even 6 gm. of the acid has been shown by the numerous experiments reported after the feeding of benzoic acid. It seems most probable that both of these acids, the *p*-hydroxylbenzoic acid and *p*-hydroxylphenylacetic acid, are at least to a large degree combined with glycocoll or some other amino-acid as a first step in the process of their elimination. This compound then undergoes a more or less complete oxidation depending on the metabolic process common to a given species of animal. In this there may be the formation of the glycocoll compound from almost any of the amino-acids as well as a partial destruction of the glycocoll compound and elimination of some of the free aromatic acid. Magnus-Levy (15) has shown that benzoylated amino-acids are not changed into hippuric acid in the animal organism but are excreted in the same form in the urine. Thierfelder and Sherwin on the contrary find phenaceturic acid in the urine of a dog after feeding phenylacetyl glutamine.

CONCLUSION.

1. After feeding a monkey *p*-hydroxylbenzoic acid 50 to 60 per cent of it was isolated from the urine as the free acid.

2. When a monkey received 1 gm. of *p*-hydroxylphenylacetic acid 32 per cent of it was found in the urine in an uncombined state. After feeding 4.5 gm., 48.37 per cent was regained from the urine in an uncombined state while 11.27 per cent occurred (in combination with glycocoll) as *p*-hydroxylphenaceturic acid.

3. *p*-Hydroxylphenaceturic acid, found in only a very small amount by Salkowski and Salkowski, was obtained in sufficient amounts for analysis and the preparation of the copper salt.

4. The process of metabolism of the aromatic amino-acids in the monkey is the same as that of the lower animals and different from that of man. The monkey excretes the *p*-hydroxylbenzoic acid in the urine in an uncombined state while a partial combination with glycocoll takes place in the human organism. The *p*-hydroxylphenylacetic acid is partly excreted as *p*-hydroxylphenaceturic acid in the case of the monkey and lower animals but is excreted in an uncombined form in the urine of man.

BIBLIOGRAPHY.

1. Sherwin, C. P., *J. Biol. Chem.*, 1917, xxxi, 307.
2. Thierfelder, H., and Sherwin, C. P., *Ber. chem. Ges.*, 1915, xlvii, 2630; *Z. physiol. Chem.*, 1915, xciv, 1. Sherwin, Inaugural Dissertation, Tübingen, 1915.
3. Hunter, A., and Givens, M. H., *J. Biol. Chem.*, 1914, xvii, 55.
4. Baumann, L., and Oviatt, E., *J. Biol. Chem.*, 1915, xxii, 43.
5. Salkowski, E., and Salkowski, H., *Z. physiol. Chem.*, 1882–83, vii, 160.
6. Baumann, E., and Herter, E., *Z. physiol. Chem.*, 1877–78, i, 244.
7. Bertagnini, C., *Ann. Chem. u. Pharm.*, 1856, xcvii, 248.
8. Maly, A, *Sitzungsber. wien. Acad.*, 1872, lxv, 39.
9. Schotten, C., *Z. physiol. Chem.*, 1882–83, vii, 27.
10. Baumann, E., *Ber. chem. Ges.*, 1880, xiii, 279.
11. Baumann, *Z. physiol. Chem.*, 1880, iv, 304.
12. Salkowski and Salkowski, *Z. physiol. Chem.*, 1882–83, vii, 171.
13. Baumann, *Z. physiol. Chem.*, 1878–79, ii, 335.
14. Magnus-Levy, A., *Biochem. Z.*, 1907, vi, 502.
15. Magnus-Levy, *Biochem. Z.*, 1907, vi, 541.

THE DETERMINATION OF TYROSINE IN PROTEINS.

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In the determination of the amino-acids in proteins, the tyrosine is usually estimated by hydrolyzing a separate 50 gm. portion of the protein in addition to the larger quantity used for the esterification. The hydrolysis may be accomplished by means of either sulfuric or hydrochloric acid. In most instances sulfuric acid has been employed. After the hydrolysis the sulfuric acid is removed quantitatively by means of barium hydroxide. This results in a large precipitate of barium sulfate which must be washed repeatedly to remove all of the tyrosine. The tyrosine, together with the other amino-acids, is thus left in a large volume of water which must be concentrated before the tyrosine will crystallize out.

When hydrochloric acid is used the hydrolysate is filtered to remove humin and the filtrate is decolorized with carbon. It is then concentrated under diminished pressure to a sirup in order to remove as much hydrochloric acid as possible. The residue is diluted to 1 liter with water. The chlorine content is determined in an aliquot part and hydrochloric acid is neutralized by adding the calculated quantity of potassium hydroxide. The final volume of solution thus obtained is less than when sulfuric acid is used and there is no precipitate to adsorb the tyrosine. This method is therefore the more convenient and less laborious.

In a recent hydrolysis of kafirin, the alcohol-soluble protein of kafir, *Andropogon sorghum* (1), determinations of tyrosine were made by both methods and the quantities found were 2.48 and 2.45 per cent, respectively. In both determinations the solutions were gradually concentrated with the removal of successive crops of tyrosine until only traces of tyrosine crystallized out, together

with considerable quantities of other amino-acids. Nevertheless, the mother liquors still gave tests for tyrosine with Millon's reagent showing that all of the tyrosine had not been isolated.

A third determination was therefore made using the colorimetric method of Folin and Denis (2) after hydrolyzing 1 gm. of kafirin by boiling for 12 hours with 25 cc. of 20 per cent hydrochloric acid. This determination indicated the presence of 5.49 per cent of tyrosine in kafirin.

When tyrosine is to be isolated from a protein it is customary to boil the protein with the mineral acid for from 18 to 30 hours or even longer. It is questionable whether it is advisable to boil for so long a period. Gortner (3) has shown that under certain conditions tyrosine is slowly decomposed when a protein is boiled with hydrochloric acid. In order to ascertain whether more tyrosine can be isolated by decreasing the duration of the hydrolysis, a 50 gm. sample of kafirin was hydrolyzed for 12 hours with 20 per cent hydrochloric acid. In this experiment 3.91 per cent of tyrosine was isolated. This is 1.43 per cent more than was obtained when kafirin was hydrolyzed for 48 hours. It therefore seems unnecessary and even detrimental to continue the hydrolysis for more than 12 hours. It is very unlikely that all of the tyrosine was isolated in this experiment since it is practically impossible to crystallize out all of the tyrosine from the hydrolysates of most proteins. It is therefore probable that the percentage of tyrosine indicated by the colorimetric method of Folin and Denis represents more accurately the quantity of tyrosine present in the protein than does the percentage obtained by direct isolation.

Objections have been raised by Abderhalden and Fuchs (4) and Abderhalden (5) to the colorimetric method of Folin and Denis on the ground that tryptophane, oxytryptophane, and *l*-oxyproline, if present, will cause high results. These authors, however, report only qualitative tests with the above amino-acids and do not consider the effect of hydrolysis. It is true that tryptophane gives a blue color with the reagent of Folin and Denis, but the intensity of the color is much less than that given by an equivalent weight of tyrosine. It is well known that tryptophane is decomposed by acid hydrolysis. To ascertain whether this decomposition is complete and that the decomposition products do not give a color with the reagent of Folin and

Denis, the following experiment was made: 5 per cent of tryptophane was added to 0.5 gm. of kafirin and the mixture was boiled with 20 per cent hydrochloric acid for 12 hours. The tyrosine in the hydrolysate was estimated by the method of Folin and Denis and was found to be 4.36 per cent. A hydrolysis of kafirin performed under similar conditions without the addition of tryptophane gave 4.84 per cent of tyrosine. Hence the tryptophane was completely decomposed and its decomposition products gave no blue color with the reagent of Folin and Denis. It is also to be expected that oxytryptophane, if present, would be decomposed by acid hydrolysis, since the presence of the hydroxyl group would probably render it less stable than tryptophane. A sample of gelatin to which tryptophane had been added was also hydrolyzed. The blue color obtained with the reagent of Folin and Denis was of the same intensity as that obtained by a control hydrolysis where no tryptophane was added. The faint blue color obtained from the hydrolysate of the gelatin was probably due to tyrosine since the gelatin gave a distinct test for tyrosine with Millon's reagent.

Abderhalden (5) states that *l*-oxyproline gives a blue color with the reagent of Folin and Denis. On the other hand, Folin and Denis (2) obtained only a faint blue color from the hydrolysate of gelatin which contains 3 to 6 (6) per cent of oxyproline. This color was probably due to tyrosine. We tested a number of high grade samples of gelatin and did not find one that did not respond to the test for tyrosine with Millon's reagent. Even gelatin prepared from carefully cleaned cartilaginous rings of ox trachea gave a decided test for tyrosine.

Tyrosine and cystine are the least soluble in water of all the amino-acids obtained from the hydrolysate of a protein. Most of the cystine present in proteins is decomposed by prolonged acid hydrolysis and does not interfere with the isolation of tyrosine from most proteins. On the other hand, the presence of considerable quantities of other amino-acids in a hydrolysate may render the tyrosine readily soluble. We encountered a case in which considerable tyrosine was separated from a hydrolysate. After this tyrosine had been removed a large crop of almost pure leucine separated. On concentrating the mother liquor considerably more tyrosine mixed with a little leucine was obtained. In an-

other instance we had a similar experience with a fraction of a hydrolysate from which the leucine and some valine had been removed and which on further concentration still yielded some pure tyrosine.

CONCLUSIONS.

The method of Folin and Denis for the determination of tyrosine has been investigated. It has been found that tryptophane is completely decomposed during the hydrolysis of proteins with hydrochloric acid and that the decomposition products do not interfere with the determination of tyrosine by the method of Folin and Denis. Since tyrosine is decomposed to some extent during hydrolysis there seems to be no advantage in hydrolyzing more than 12 hours. It has been shown that oxyproline does not interfere with the determination of tyrosine by the method of Folin and Denis. Gelatin which is said to contain up to 6 per cent of oxyproline gave but little color with the reagent of Folin and Denis after hydrolysis. This color was probably due to tyrosine in the gelatin since a test for tyrosine was obtained by Millon's reagent.

BIBLIOGRAPHY.

1. Johns, C. O., and Brewster, J. F., *J. Biol. Chem.*, 1916–17, xxviii, 59.
2. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 245.
3. Gortner, R. A., *J. Biol. Chem.*, 1916, xxvi, 200.
4. Abderhalden, E., and Fuchs, D., *Z. physiol. Chem.*, 1913, lxxxiii, 468.
5. Abderhalden, E., *Z. physiol. Chem.*, 1913, lxxxv, 91.
6. Plimmer, R. H. A., The chemical constitution of the proteins, London, 3rd edition, 1917, pt. 1, 124.

THE HYDROLYSIS OF KAFIRIN.

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Kafirin is the alcohol-soluble protein of kafir, *Andropogon sorghum*. The sample used in this investigation was prepared according to the method previously published (1). The methods employed in the hydrolysis of kafirin are essentially those devised by Fischer and modified by Osborne. Certain modifications recommended by other investigators were found helpful and are noted below.

The esterification of the amino-acids was accomplished by the method of Phelps and Phelps (2, 3). The esters were liberated from their hydrochlorides with sodium ethylate instead of sodium hydroxide. This change was suggested by Abderhalden and Rostoski (4). Instead of distilling the esters directly after filtering off sodium chloride and evaporating the alcohol, the esters were mixed with water and extracted with ether. This made it possible to make a second esterification of the portion not soluble in ether. Since a 3 per cent solution of sodium ethylate in alcohol was used to liberate the esters from their hydrochlorides, the free esters were left dissolved in a large volume of alcohol. When this was distilled off, some of the esters were carried over. These were recovered by adding hydrochloric acid to the receiver and finally evaporating the alcohol. The ether with which the esters had been extracted also carried over esters equivalent to 12 gm. of amino-acids. These were recovered by acidifying the ether with hydrochloric acid and evaporating the ether after the mixture had stood for some weeks to allow any glycine hydrochloride that might be present to separate. Osborne and Liddle (5) have shown that other esters as well as that of glycine can be carried over with the ether.

Tyrosine was first determined in the usual manner by making separate hydrolyses of 50 gm. samples of the protein. Two determinations were made, using sulfuric acid for one hydrolysis and hydrochloric (6) acid for another. These methods gave similar results; namely, 2.45 and 2.48 per cent, respectively. Since it is practically impossible to crystallize all of the tyrosine from a solution of the hydrolysate of a protein, it seemed probable that these results were low. A third determination of tyrosine was therefore made, using the colorimetric method of Folin and Denis (7, 8). This determination indicated the presence of 5.49 per cent of tyrosine in kafirin, or more than twice as much as was obtained by direct crystallization in the first two determinations. A fourth determination of tyrosine was made by hydrolyzing the kafirin with hydrochloric acid for only 12 hours and crystallizing in the usual manner. This determination gave 3.91 per cent of pure tyrosine.

The determination of aspartic acid was made by the method of Foreman (9) on a separate 50 gm. portion of the protein. Much higher results were obtained by this method than by separating the aspartic acid from the esters.

The method of Van Slyke and Levene (10) for the separation of leucine and valine was employed and found to be very satisfactory.

The percentages of the basic amino-acids in kafirin, as determined by the Van Slyke method have already been published (1). Two new determinations were made by Mr. A. J. Finks of this Laboratory, and they agreed well with the previous results. The percentages of the different amino-acids obtained in the hydrolysis of kafirin have been summarized in Table I. The values recorded in this table for leucine, proline, and glutaminic acid are somewhat higher than the figures given in the experimental work here recorded. They were obtained by a method of isolation which is now under investigation in this Laboratory. Since kafirin resembles zein, the alcohol-soluble protein of maize, in many respects, the percentages of amino-acid found in zein are also given for the sake of comparison. The values given for zein are compiled from the results of several hydrolyses and represent the highest reliable values recorded.

TABLE I.

Percentage of Amino-Acids in Kafirin and Zein.

	Kafirin.	Zein.*
	per cent	*per cent*
Glycine	0.00	0.00
Alanine	8.08	9.79
Valine	4.26	1.88
Leucine	15.44	19.55
Proline	7.80	9.04
Phenylalanine	2.34	6.55
Aspartic acid	2.27	1.71
Glutaminic acid	21.23	26.17
Serine	—	1.02
Oxyproline	—	—
Tyrosine	5.49	3.55
Cystine	0.84	—
Arginine	1.59	1.55
Histidine	1.12	0.82
Lysine	0.95	0.00
Tryptophane	Present.	0.00
Ammonia	3.46	3.64
Total	74.87	85.27

* Osborne, T. B., and Liddle, L. M., *Am. J. Physiol.*, 1910, xxvi, 304.

EXPERIMENTAL.

500 gm. of the kafirin, equivalent to 473 gm. of ash- and moisture-free protein were hydrolyzed in three separate portions of 100, 200, and 200 gm. each by heating with 1,500 cc. of hydrochloric acid (specific gravity 1.1) on a steam bath until nearly all of the substance had gone into solution, and then boiling for 40 hours in an oil bath.

Glutaminic acid was separated from each portion by concentrating the solutions to about two-thirds of the original volumes, saturating with hydrochloric acid gas at 5–10°C., and allowing them to stand in a refrigerator for 5 to 6 days. The crystalline product was filtered off by suction on asbestos, washed with cold alcohol which had been saturated with dry hydrochloric acid gas, dissolved in water, and most of the color removed by means of

animal charcoal. The solutions were then boiled with an excess of barium hydroxide until nearly all of the ammonia had been expelled. The barium hydroxide was removed quantitatively with sulfuric acid. From the resulting solutions there were obtained 23.30, 42.97, and 49.15 gm. respectively of practically pure glutaminic acid hydrochloride, equivalent to 92.50 gm. of the free acid, or 19.55 per cent of the kafirin. The glutaminic acid hydrochloride gave the following analytical results:

Carbon and Hydrogen.—0.3487 gm. substance gave 0.4167 gm. CO_2 and 0.1769 gm. H_2O.

Nitrogen.—0.1825 gm. substance required 10.3 cc. 0.1 N H_2SO_4.

Chlorine.—0.2028 gm. substance gave 0.1580 gm. AgCl.

	C	H	N	Cl
Calculated for $C_5H_9O_4N.HCl$, *per cent*	32.70	5.49	7.63	19.32
Found, *per cent*	32.59	5.68	7.70	19.26

The filtrates from the glutaminic acid hydrochloride were united and concentrated under diminished pressure to a thick sirup, which was taken up in absolute alcohol and again concentrated to remove water. The residual amino-acid hydrochlorides were then esterified according to the method of Phelps and Phelps (2) as follows: The sirup was dissolved in 50 cc. of concentrated alcoholic hydrochloric acid and 50 cc. of absolute alcohol. After adding 40 gm. of fused zinc chloride, the vapors of 3,500 cc. of absolute alcohol containing 105 cc. of alcoholic hydrochloric acid were passed, during 11 hours, through the solution which was kept at a temperature of 105–110°C. by heating in an oil bath. The esters were dissolved in about a liter of absolute alcohol. After standing over night about 32 gm. of ammonium chloride had separated, which was filtered off, washed with absolute alcohol, and the washings and the solution of esters were united, and made up to 2 liters with absolute alcohol. Chlorine was determined in two aliquot portions of 20 cc. each. The esters were liberated from their hydrochlorides by the addition of the calculated quantity of sodium dissolved in absolute alcohol so as to make about a 3 per cent solution of sodium ethylate. The sodium chloride was removed by decantation and centrifugation, and the alcohol by distillation at about 40°C. under diminished pressure. The alcohol was saved for further exami-

nation. The thick, sirupy residue of esters was dissolved in about 500 cc. of ice cold water, and the solution shaken out several times with ether. Finally solid potassium carbonate was added to the residue until a thick paste was obtained, and the mixture extracted with ether until the ether extracts were colorless, the temperature being kept below 0°C. The ether solutions of the esters were dried in the usual way by standing over anhydrous sodium sulfate.

The residue containing the potassium carbonate was freed from inorganic salts by dissolving in water and saturating with hydrochloric acid gas and concentrating on the steam bath and filtering off successive crops of salts. The salts removed were washed with absolute alcohol and the washings added to the mother liquor. This solution was then esterified as described above.

The ether was removed from the united esters by distillation at atmospheric pressure and 320 gm. of esters were obtained.

Ether Distilled from the Esters.—The ether which was distilled from the esters was acidified with alcoholic hydrochloric acid and allowed to stand for 3 to 4 weeks. There was no separation of glycine ester hydrochloride crystals. The ether was distilled off, the residue taken up in water, an excess of barium hydroxide added, and the solution boiled for 6 hours to hydrolyze the esters and remove ammonia. After removing the barium hydroxide and chlorine the solution was evaporated to dryness. Proline was extracted from the residual amino-acids with absolute alcohol. There was thus obtained 10.87 gm. of amino-acids insoluble in alcohol, which were added to the fraction similarly obtained from Fraction I of the distilled esters (see below).

The alcoholic proline extract was evaporated to dryness, taken up in water, and examined according to the method of Van Slyke for determining amino nitrogen. The total nitrogen was 0.1825 gm., the amino nitrogen, 0.06468 gm., making the difference, 0.1178 gm. which corresponds to 0.97 gm. of proline.

Alcohol Distilled from the Esters at 40°C.—The alcohol, which was distilled from the esters after their liberation from their hydrochlorides by means of sodium ethylate, was acidified with alcoholic hydrochloric acid and the solution concentrated to a thick sirup. 5 volumes of water were added and the esters saponified by boiling from 6 to 7 hours. The chlorine was removed

with silver sulfate and the solution evaporated to dryness. After removing the proline in the usual way, there remained 19 gm. of amino-acids insoluble in alcohol. The alcoholic extract of proline was added to that similarly obtained from Fraction I (see below).

The amino-acids insoluble in alcohol when subjected to fractional crystallization yielded 2.21 gm. of leucine and 16.03 gm. of alanine. Analysis showed the leucine to have the following composition:

Carbon and Hydrogen.—0.1021 gm. substance gave 0.2049 gm. CO_2 and 0.0927 gm. H_2O.

	C	H
Calculated for $C_6H_{13}NO_2$, *per cent*	54.96	9.99
Found, *per cent*	54.73	10.16

The alanine gave the following results when analyzed:

Carbon and Hydrogen.—0.2001 gm. substance gave 0.2969 gm. CO_2 and 0.1422 gm. H_2O.

	C	H
Calculated for $C_3H_7O_2N$, *per cent*	40.41	7.92
Found, *per cent*	40.47	7.95

The esters of the amino-acids after removal of the ether were distilled as follows, using liquid air to condense vapors:

Fraction.	Temperature of the vapors up to	Pressure.	Weight.
	°C.	*mm.*	*gm.*
I	92	15.0	113.5
II	115	0.46	38.0
Undistilled residue			128.0

Fraction I.—This fraction was saponified by boiling for about 8 hours with 10 volumes of water. The solution was evaporated to dryness and again taken down several times with absolute alcohol to remove water. To the amino-acids of this fraction were added those obtained from the ether distilled from the esters, together with 1 gm. of substance which had separated on long standing from the alcoholic extracts of proline. Proline was extracted by boiling with absolute alcohol. The amino-acids insoluble in

alcohol, which weighed 71 gm., were subjected to fractional crystallization. There were obtained 25 gm. of leucine which had the following composition:

Carbon and Hydrogen.—0.2088 gm. substance gave 0.4209 gm. CO_2 and 0.1913 gm. H_2O.

	C	H
Calculated for $C_6H_{13}NO_2$, *per cent*	54.96	9.99
Found, *per cent*	54.98	10.25

There was further obtained a fraction weighing 30 gm. consisting of a mixture of leucine and valine. These two acids were separated by means of their lead salts according to the method of Van Slyke and Levene (10). The lead leucine, which weighed 32 gm., equivalent to 17.82 gm. of leucine, gave the following results on analysis:

Lead.—0.2914 gm. substance gave 0.1889 gm. $PbSO_4$.

	Pb
Calculated for $Pb(C_6H_{12}O_2N_2)_2$, *per cent*	44.29
Found, *per cent*	44.27

The filtrate from the lead leucine, after removal of the lead with hydrogen sulfide, was evaporated to dryness. The residue, after removal of acetic acid and ammonium acetate with the ether-alcohol mixture, weighed 11.69 gm. and was practically pure valine. Without having been recrystallized it gave the following results on analysis:

Carbon and Hydrogen.—0.2826 gm. substance gave 0.5284 gm. CO_2 and 0.2406 gm. H_2O.

	C	H
Calculated for $C_5H_{11}O_2N$, *per cent*	51.28	9.47
Found, *per cent*	51.00	9.53

The remainder of Fraction I, which weighed 28.36 gm., was separated into nine fractions. The ninth fraction which contained most of the remaining substance, contained 41.25 per cent of carbon indicating the presence of alanine contaminated with valine. This percentage of carbon also shows that there was little or no glycine present. After extensive fractional crystallization it was found impossible to effect a separation of the alanine and valine. The fractions were accordingly united and thoroughly mixed. Analysis showed it to contain 43.34 per cent of

carbon and 7.86 per cent of hydrogen, corresponding to 7.66 gm. of valine and 20.67 gm. of alanine in this mixture.

The united alcoholic extracts containing the proline, after standing for several weeks, were filtered from a small amount of substance which had separated out, and the filtrate was evaporated to dryness; the sirupy residue was dissolved in water and made up to 500 cc. The total nitrogen in this solution was 2.3090 gm., the amino nitrogen, 0.3273 gm., the difference, 1.9817 gm., corresponding to 16.29 gm. of proline. The proline contained in the remainder of the solution after having made the Van Slyke amino nitrogen determination, was isolated and weighed in the form of its copper salts. These contained 10.88 gm. of *l*-proline and 5.79 gm. of *dl*-proline, which after correcting for the losses involved in the Van Slyke analyses amounts to 19.19 gm. of proline.

The *l*-proline copper salt was converted into the free acid and identified in the form of the phenylhydantoin which crystallized in beautiful characteristic prisms, melting sharply at 143°C.

The air-dried *dl*-copper salt showed the following composition on analysis:

Water.—0.3449 gm. substance lost 0.0377 gm. H_2O at 110°C.

	H_2O
Calculated for $C_{10}H_{16}O_4N_2Cu2H_2O$, *per cent*	10.99
Found, *per cent*	10.93

Copper.—0.2843 gm. substance dried at 110° gave 0.0770 gm. CuO.

	Cu
Calculated for $C_{10}H_{16}O_4N_2Cu$, *per cent*	21.81
Found, *per cent*	21.64

Fraction II.—The phenylalanine ester was extracted from this fraction with ether in the usual way and saponified by heating with concentrated hydrochloric acid. The resulting product was united with that obtained in a similar way from the undistilled residue.

The aqueous layer which remained after the extraction with ether was saponified by boiling with barium hydroxide, and 2.72 gm. of aspartic acid were isolated in the form of the barium salt. Analysis of the free aspartic acid obtained by decomposing the barium salt with sulfuric acid showed it to have the following composition:

Carbon and Hydrogen.—0.1644 gm. substance gave 0.2191 gm. CO_2 and 0.0810 gm. H_2O.

	C	H
Calculated for $C_4H_7O_4N$, *per cent*	36.09	5.26
Found, *per cent*	36.35	5.51

No glutaminic acid or copper aspartate could be obtained from the filtrate from the barium aspartate.

Distillation Residue.—The undistilled portion of the esters was mixed with 5 volumes of water and the mixture shaken in the usual way with an equal volume of ether. On standing there separated from the ether solution about 4 gm. of diketopiperazines which were filtered off and the ether was allowed to evaporate at room temperature. The residual esters were hydrolyzed in the usual way with concentrated hydrochloric acid. After removing a small amount of oil by extraction with ether, the dark, red solution was largely decolorized by treatment with animal charcoal. To this solution was added that similarly obtained from Fraction II.

On account of an admixture with the phenylalanine contained in this solution, of considerable leucine and a little tyrosine, chlorine was removed quantitatively from the mixture of amino-acid hydrochlorides and the whole subjected to fractional crystallization. The total amount of amino-acids thus obtained was equivalent to 10.65 gm. of phenylalanine, 19.51 gm. of leucine, and 2.38 gm. of tyrosine. The phenylalanine on analysis gave the following results:

Carbon and Hydrogen.—0.1743 gm. substance gave 0.4168 gm. CO_2 and 0.1079 gm. H_2O.

	C	H
Calculated for $C_9H_{11}O_2N$, *per cent*	65.45	6.66
Found, *per cent*	65.22	6.93

The leucine had the following composition:

Carbon and Hydrogen.—0.2536 gm. substance gave 0.5096 gm. CO_2 and 0.2241 gm. H_2O.

	C	H
Calculated for $C_6H_{13}NO_2$, *per cent*	54.96	9.99
Found, *per cent*	54.80	9.88

The aqueous layer remaining after the extraction with ether was saponified with barium hydroxide, and the latter removed

quantitatively with sulfuric acid. The solution was then concentrated and saturated with hydrochloric acid gas at a low temperature. On standing there separated 8.09 gm. of glutaminic acid hydrochloride. The free acid obtained by decomposing the hydrochloride with an equivalent amount of normal potassium hydroxide was analyzed with the following results:

Carbon and Hydrogen.—0.2291 gm. substance gave 0.3439 gm. CO_2 and 0.1285 gm. H_2O.

	C	H
Calculated for $C_5H_9O_4N$, *per cent*	40.81	6.12
Found, *per cent*	40.94	6.28

No copper aspartate could be obtained from the filtrate from the glutaminic acid hydrochloride.

Aspartic Acid.—47.27 gm. of ash- and moisture-free protein were hydrolyzed by boiling with 200 cc. of hydrochloric acid (specific gravity 1.1) for 50 hours and the aspartic acid was determined according to Foreman's method (9) by its separation, together with glutaminic acid, from the other products of hydrolysis, as the calcium salts. There were isolated 6.86 gm. of glutaminic acid in the form of the hydrochloride, and 2.22 gm. of copper aspartate equivalent to 1.07 gm. of the free acid. Analysis showed the copper aspartate to have the following composition:

Copper.—0.2061 gm. substance, air-dried, gave 0.0602 gm. CuO.

	Cu
Calculated for $C_4H_5O_4NCu.4\frac{1}{2}H_2O$, *per cent*	23.07
Found, *per cent*	23.34

Tyrosine.—Four different determinations of tyrosine were made. A quantity equivalent to 47.27 gm. of ash- and moisture-free protein was hydrolyzed by boiling with 100 cc. of concentrated hydrochloric acid and 100 cc. of water for 48 hours. The solution was then concentrated under diminished pressure and again taken down two or three times with water to remove as much as possible of the hydrochloric acid. The residual sirup was dissolved in water, decolorized with charcoal, and made up to 1,000 cc. The amount of chlorine in the solution was determined and the calculated amount of normal potassium hydroxide added. By concentration of the solution there were obtained 1.17 gm. of

tyrosine equivalent to 2.48 per cent, which gave the following results when analyzed:

Carbon and Hydrogen.—0.1527 gm. substance gave 0.3350 gm. CO_2 and 0.0868 gm. H_2O.

	C	H
Calculated for $C_9H_{11}O_3N$, *per cent*	59.67	6.08
Found, *per cent*	59.83	6.36

A second determination made by hydrolyzing 47.30 gm. of the ash- and moisture-free protein by boiling for 35 hours with 150 gm. of sulfuric acid and 300 cc. of water, removing the sulfuric acid quantitatively with barium hydroxide, and concentrating to crystallization, gave 1.18 gm. of tyrosine equivalent to 2.45 per cent of the protein.

The third determination was made colorimetrically according to the method of Folin and Denis (7). A quantity of kafirin equivalent to 1 gm. of the ash- and moisture-free protein was hydrolyzed by boiling for 12 hours with 25 cc. of 20 per cent hydrochloric acid. The solution was decolorized with charcoal and made up to 100 cc. The tyrosine in the solution determined colorimetrically as described by Folin and Denis, was equivalent to 5.49 per cent of the protein.

A fourth determination of tyrosine was made as follows: A quantity of kafirin equivalent to 46.81 gm. of ash- and moisture-free protein was hydrolyzed for a period of only 12 hours by boiling with 200 cc. of 20 per cent hydrochloric acid. After removing as much as possible of the free hydrochloric acid by concentrating under diminished pressure, the residual hydrochloric acid was neutralized by adding the calculated quantity of normal potassium hydroxide. It was then possible to crystallize out 1.83 gm. of tyrosine which are equivalent to 3.91 per cent of the kafirin. This is 1.43 per cent higher than the best yield obtained by direct isolation in the experiments described above in which the protein was hydrolyzed for 48 hours.

BIBLIOGRAPHY.

1. Johns, C. O., and Brewster, J. F., *J. Biol. Chem.*, 1916–17, xxviii, 59.
2. Phelps, I. K., and Phelps, M. A., *Am. J. Sc.*, 1907, xxiv, 194.
3. Osborne, T. B., and Jones, D. B., *Am. J. Physiol.*, 1910, xxvi, 212.
4. Abderhalden, E., and Rostoski, O., *Z. physiol. Chem.*, 1905, xlvi, 125.

5. Osborne, T. B., and Liddle, L. M., *Am. J. Physiol.*, 1910, xxvi, 295.
6. Abderhalden, E., and Teruuchi, Y., *Z. physiol. Chem.*, 1906, xlviii, 528.
7. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 245.
8. Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1918, xxxvi, 319.
9. Foreman, F. W., *Biochem. J.*, 1914–15, viii, 463.
10. Van Slyke, D. D., and Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1908–09, vi, 54.

THE QUANTITATIVE DETERMINATION OF PHOSPHORUS BY THE NEPHELOMETRIC METHOD.*

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The nephelometric method of phosphorus determination proposed by Pouget and Chouchak[1] has since been developed and used by Kober and Egerer,[2] Greenwald,[3] and Bloor.[4] In determinations made by this method, the phosphate is precipitated from its solutions by what I shall call the "strychnine molybdate reagent." This is prepared by treating a solution of sodium molybdate in hydrochloric acid and water with strychnine sulfate. The method of preparing the reagent and the special procedure by which the phosphate is precipitated will be described more fully later. The determinations which form the basis for this article were made with the Duboscq colorimeter converted to a nephelometer, as described by Bloor.[5]

For convenience of discussion the density of a phosphate suspension may be defined as a quantity proportional to the amount of phosphate contained in the solution from which it was precipitated; and its nephelometric value, as the amount of light which a given volume of it will reflect to the eye of the observer under the conditions to which it is subjected in the nephelometer. It is obvious that there will be no proportionality between the densities and the nephelometric values of phosphate suspensions

* Published by permission of the Secretary of Agriculture.

[1] Pouget, I., and Chouchak, D., *Bull. Soc. chim. France*, 1909, v, 104; 1911, ix, 649.

[2] Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

[3] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 29.

[4] Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 133, 145; 1916, xxiv, 452.

[5] Bloor, *J. Biol. Chem.*, 1915, xxii, 145.

except under certain very special conditions. For the same quantity of precipitated phosphate may be divided into a few large particles or into more numerous smaller ones; the nephelometric value will obviously be different in the two cases, though the density may be the same. For this reason the investigators who have made use of the nephelometric method have laid great emphasis on the necessity of precipitating the standard and test suspensions as nearly as possible at the same time and under the same conditions.

When this is done, the nephelometric values of different phosphate suspensions are roughly proportional to their densities. By this I mean that if two suspensions of different densities are compared in the nephelometer under proper precautions, it will be found that the lengths of column which must be exposed to the light in order to make the illumination equal in the two semicircles are roughly inversely proportional to the densities. If, for example, the two suspensions have densities of 9 and 10 respectively, and if a column of the former 30 mm. long is exposed to the light, it will be found that approximately 27 mm. of a similar column of the latter must be exposed in order to make the illumination equal in the two semicircles.

But the proportionality between difference in density and difference in nephelometric value is not exact; and it is a very important practical question whether the correction which must be made depends only on fixed factors, such as the constructional peculiarities of the nephelometer and the degree of difference between the two suspensions to be compared, or whether it depends also on variable factors, such as the temperature at which the two suspensions are made and the character of the reagent used in precipitating them.

Kober and Egerer and Bloor imply in their discussions of the method that they think the former alternative is the case. Thus Kober and Egerer give mathematical formulas to be used in calculating the density of suspensions from the readings given by them in the nephelometer;[6] and Bloor says:[7]

[6] Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2378, 2379.

[7] Bloor, *J. Biol. Chem.*, 1915, xxii, 143.

"Since the readings obtained from different solutions are not exactly proportional to the amount of phosphate present and since the differences between the observed and theoretical reading increase as the differences in phosphate content increase it is necessary to calibrate the instrument for different strengths of test solution and for different standards, although where the test solution does not differ from the standard by more than 25 per cent the corrections fall within the limit of error of the determination and no correction can be made."

My own experience leads me to dissent from the view implied in the quotations given above, though I still think that the nephelometric method is of great value. The importance of a method by which 0.03 mg. of phosphorus can be quantitatively determined with even approximate accuracy is so obvious, that I feel justified in reporting my experience in some detail.

I have had occasion to make several hundred nephelometric determinations of the phosphorus content of known solutions. Of these, perhaps 50 per cent fell within 5 per cent of what was known to be the correct value; the others were off, sometimes as much as 30 per cent. There were two possible ways of accounting for the incorrect results. One was to suppose that they were due to accident—unconscious mistakes, contaminations by volatile materials in the air of the laboratory, by dust, etc. Another was to suppose that they were due to influences which are controllable, but which had not yet been controlled; such, for instance, as the temperature prevailing during various parts of the procedure and the age of the reagents.

My subsequent experience shows that temperature and the character of the reagent as determined by its age and by other factors are matters of the greatest importance. Very large deviations from what may be called the theoretical readings may be caused by the character of the reagent and by the amount of hydrochloric acid present at the time the phosphate suspensions are made, and it will therefore be necessary to consider these matters in some detail.

My strychnine molybdate reagent has been made up according to the description given by Kober and Egerer.[8] Specially pre-

[8] Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2374–2376.

pared sodium molybdate,[9] water, and hydrochloric acid are mixed together in certain proportions, and to this solution a small amount of a saturated strychnine sulfate solution is added. The filtrate obtained from the resulting precipitation is used as the reagent. My determinations have generally been made according to the procedure given by Bloor.[10] 25 cc. of water are placed in each of two 50 cc. flasks, and 5 cc. of "1: 1 HCl" and 5 cc. of the reagent are added in each case. 10 cc. of the standard phosphate solution are then run into one of the flasks, and 10 cc. of the test solution, into the other; the resulting suspensions are made up to volume with water, and subsequently read against each other in the nephelometer.

Kober and Egerer assert that the strychnine molybdate reagent as made up by them with hydrochloric acid is stable and gives quantitative and constant results.[11] This statement is not in entire accord with my experience. I have found that the stability and other important qualities of the reagent depend, to a surprising extent, on the strength of hydrochloric acid used in preparing it, and this concentration should be known with great exactness.

Both Kober and Egerer and Bloor use the phrase "1: 1 hydrochloric acid" in the descriptions of their procedures, by which they mean acid made by adding together about equal parts by volume of the "concentrated hydrochloric acid" which is supplied

[9] The sodium molybdate was in most cases prepared as described by Kober and Egerer from Merck's molybdic acid "highest purity." The product obtained from this preparation is pure white crystalline powder, 15 gm. of which readily dissolve in 30 cc. of water, leaving no residue. Reagents made from such sodium molybdate are colorless when made, and remain so indefinitely. Lately, I have been unable to obtain Merck's molybdic acid, and have used a Baker and Adamson preparation, which is stated on its bottle to contain 12 to 15 per cent of ammonium nitrate. Reagents made from this preparation can be used fairly satisfactorily, though they are somewhat yellow when made and turn a deeper yellow on standing, probably on account of the nitric acid present.

The great majority of the nephelometric determinations and all the experiments reported in this article were carried out with reagents made from the Merck preparations, and the conclusions formed in this article are all drawn from experiments in which the Merck preparation was used.

[10] Bloor, *J. Biol. Chem.*, 1916, xxiv, 452.

[11] Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2374.

commercially, and water. In the subsequent discussion I shall give the concentration of solutions in terms of per cent by weight, unless otherwise indicated. By a "20 per cent solution of hydrochloric acid," for instance, a solution, which contains 20 gm. of HCl and 80 gm. of water, is indicated.

The chemically pure concentrated hydrochloric acid which we have obtained is advertised on its bottles to contain 37 per cent HCl. As a matter of fact, however, it has generally contained only about 35 per cent. Differences of this order in the concentration of hydrochloric acid used produce very marked differences in the character of the resulting strychnine molybdate reagent.

One gathers from the figures given by Kober and Egerer that their "1:1 hydrochloric acid" was of various strengths. In their article,[12] for instance, they direct that the 1:1 HCl be made by diluting 50 cc. of HCl with a specific gravity of 1.20 to 100 cc. with water. On the other hand, they state[11] that their 1:1 HCl had a specific gravity of 1.098. If it may be supposed that the specific gravities were taken at 15°, as is customary, the first solution would have a concentration of over 21 per cent, and the second, of 19.5 per cent. If the strychnine molybdate reagent is made up with 1:1 hydrochloric acid containing 21 per cent HCl and if the determination is carried out as Bloor directs,[10] no precipitate at all is obtained with such amounts of phosphate as are used by Kober and Egerer and by Bloor in their determinations.

To carry out the determinations as Bloor directs, therefore, it is necessary to use 1:1 hydrochloric acid weaker than 21 per cent. I have made up batches of reagent with various strengths of HCl ranging from about 16 to 21 per cent. The weaker the hydrochloric acid used, the less is the amount of the material precipitated from the solution of sodium molybdate in HCl and water by the addition of the strychnine sulfate solution. Reagents made up with weak HCl are unstable in the sense that a precipitate forms in them spontaneously on standing. In reagents made up with stronger 1:1 HCl more material is precipitated when the strychnine is added, and the spontaneous precipitate forms more slowly. But in all reagents which can be

[12] Kober and Egerer, *J. Am. Chem. Soc.*, 1915, xxxvii, 2375.

used in carrying out the Bloor procedure, so far as my experience goes, the spontaneous precipitate does form; it is only a question of its being formed more or less rapidly. Another change takes place gradually in all the reagents, which will be fully described below.

Reagents made up with weak hydrochloric acid tend to exaggerate the difference in nephelometric value as between phosphate suspensions with a given difference in density, whereas reagents made up with strong hydrochloric acid tend to minimize the nephelometric differences. As examples of the truth of this statement, the following experiments may be given, of which the results are shown in Table I.

Two reagents were made up in exactly the same way, except that 16.15 per cent HCl was used for one, and 19.10 per cent HCl for the other. Phosphate suspensions of different densities were then precipitated according to Bloor's procedure and read against each other, first one and then the other reagent being used in making the pairs of suspensions. The same pairs of phosphorus solutions were used in each of the three cases in making up the pairs of suspensions read against each other with the different reagents. The same sort of statement applies to all the other experiments reported in this article. In each case given amounts of the same pair of phosphorus solutions were precipitated first with one reagent and then with another, or first at one temperature and then at another temperature. The experiments, therefore, demonstrate in the strictest possible manner that the discrepancies between densities and nephelometric values were the result of the character of the reagent used or of the temperature at which the suspensions were made. They could not have been due to any accidents or mistakes in making up the phosphate solutions.

The results given in Table I bring out two interesting points. These points have been confirmed in other experiments which it has not been thought worth while to publish in detail. For convenience in discussing them, it may be said that by the theoretical nephelometric readings is understood readings which would indicate an exact proportionality between the densities of the suspensions and their nephelometric values, these terms being used in the senses indicated on pages 333 and 334.

Perhaps the most important point brought out in Table I is the fact that the differences in nephelometric value may be either greater or less than the differences in density. When the 0.000095 and 0.000114 per cent solutions are precipitated with the reagent containing weak HCl, the difference in nephelometric value is exaggerated somewhat beyond the theoretical; whereas, in the case of the 0.000133 and 0.000152 per cent solutions precipitated with the other reagent, the difference in nephelometric

TABLE I.

	P concentrations in solutions from which suspensions were made	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	per cent		*mm.*	
Reagent made up with strong HCl.	0.000095 / 0.000114	0.83	30.0 / 25.2	0.84
Reagent made up with weak HCl.	0.000095 / 0.000114	0.83	30.0 / 23.9	0.80
Reagent made up with strong HCl.	0.000114 / 0.000133	0.86	30.0 / 26.9	0.90
Reagent made up with weak HCl.	0.000114 / 0.000133	0.86	30.0 / 24.6	0.82
Reagent made up with strong HCl.	0.000133 / 0.000152	0.87	30.0 / 28.9	0.96
Reagent made up with weak HCl.	0.000133 / 0.000152	0.87	30.0 / 26.4	0.88

value is very markedly minimized. A formula totally different from that given by Kober and Egerer would be needed to cover the former of these two cases. It is true that the hydrochloric acid used was considerably weaker than that recommended by these authors; but, in the latter of the two cases, where the hydrochloric acid used approached closely to one of the strengths recommended by them, the difference in nephelometric value was considerably more minimized than it should have been according to their formula. It is to be noted that the readings

obtained with the reagent containing less HCl are, on the average, closer to the theoretical than the others. On the whole, I have found it better to make up my reagents with 1:1 hydrochloric acid which contains less than 19 per cent of HCl.

Table I also shows that, other things being equal, the difference in nephelometric value between a pair of suspensions is more greatly minimized the greater their absolute density. This point is very well brought out in the case of the three pairs of suspensions precipitated by the reagent containing the strong acid. Where the densities are both low, the nephelometric readings are very near the theoretical; the nephelometric differences are somewhat minimized in the intermediate pair of suspensions; and very markedly so in the densest pair.

The situation illustrated in Table I may be summed up by saying that if the densities of phosphate suspensions are to be calculated from their nephelometric values by means of mathematical formulas, a different formula will be required for every different reagent used and for every different density of standard suspension.

If the HCl-Na_2MoO_4 solution contains a still less concentration of HCl when the strychnine sulfate is added to it in making up the reagent, the differences in nephelometric value as between phosphate suspensions of moderately different densities may be much more exaggerated than in the examples which have just been given. Following a suggestion of Dr. Bloor, I made up a batch of reagent in a manner quite different from that which has been used as a matter of routine. In this case 15 gm. of Na_2MoO_4 were dissolved in 30 cc. of H_2O, and there were then added 230 cc. of 19.10 per cent HCl, 650 cc. of H_2O, and 20 cc. of saturated strychnine sulfate solution. In making phosphate suspensions with this reagent 10 cc. of the dilute phosphate solution were added directly to 30 cc. of the reagent. The results are given in Table II.

The character of the reagent may also be markedly influenced by the temperature at which the HCl-Na_2MoO_4 solution is precipitated by adding the strychnine sulfate solution. If this precipitation is carried out at a high temperature, the resulting reagent will exaggerate the differences in nephelometric value as between phosphate suspensions of different densities. Table III shows the high degree to which this exaggeration may attain.

In all the reagents with which I have had any experience, a precipitate tends to be formed gradually, and another change goes on. This latter change consists in a growing tendency to minimize the difference in nephelometric value as between phosphate suspensions of different densities. All my reagents have tended to reach a state as a result of which the phosphate suspensions precipitated by them show no differences in nephelo-

TABLE II.

	P concentrations in solutions from which suspensions were made.	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	per cent		*mm.*	
Reagent made up as described (p. 340).	0.000095 / 0.000114	0.83	30.0 / 20.6	0.69
Reagent made up by routine method.	0.000095 / 0.000114	0.83	30.0 / 25.8	0.86

TABLE III.

	P concentrations in solutions from which suspensions were made.	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	per cent		*mm.*	
Reagent precipitated at about 45°.	0.000095 / 0.000114	0.83	30.0 / 18.0	0.60
Reagent precipitated at room temperature (about 20°).	0.000095 / 0.000114	0.83	30.0 / 25.8	0.86

metric value, even though the solutions from which they were made contained quite different concentrations of phosphate. This change often goes on slowly; the discrepancies between the actual and the theoretical nephelometric readings may not reach a stage where they destroy the value of the determinations for several weeks.

My experience with the strychnine molybdate reagent may be summed up as follows: The greater the concentration of hydrochloric acid present when the strychnine sulfate is added, the

greater will be the amount of material precipitated at that time and the greater will be the stability of the reagent. Reagents made up with strong hydrochloric acid, however, tend to minimize the differences in nephelometric value as between phosphate solutions of different densities; none of the reagents that are serviceable when used in the procedure described by Bloor are entirely stable; they all tend to form a spontaneous precipitate and to reach a state in which they show no difference in nephelometric value as between phosphate suspensions of different densities.

The temperature at which the strychnine sulfate is added to the Na_2MoO_4-HCl solution in making up the reagent may have a considerable effect on its character.

There is still another factor which may cause a lack of proportionality between differences in the densities of suspensions and the differences in their nephelometric values; namely, the temperature at which the phosphate suspensions are precipitated. It is, of course, to be expected that if phosphate suspensions are made from equally concentrated phosphate solutions but at different temperatures, they will have different nephelometric values. But the point to be made here is somewhat different from this. In this case, two suspensions are made from solutions of different concentrations, both at a given temperature. Later two other suspensions are made from the same two solutions, but both at some other temperature. Under such circumstances it will be found that the nephelometric difference between the suspensions is minimized at the higher temperature. Table IV gives an example of the kind of results that are obtained in this way.

Though the results which have been reported contribute little to the theory of nephelometric values, they show that in making such determinations it is not sufficient to assure oneself that the standard and test solutions are precipitated at the same time and under the same conditions. Even though these requirements may be ideally realized, the relation between difference in density and difference in nephelometric value may vary widely under the influence of a number of factors, of which the most important is the character of the reagent used. To make anything like a complete study of these factors would require great care and much time, for they react on each other in such a way

as to make it very difficult to disentangle their individual influences. But by taking certain precautions, the nephelometric method can be used in a practical way, even with our present scanty knowledge regarding it.

It has been a practically invariable rule in my experience that, whatever the relation between difference in density and difference in nephelometric value, less dense solutions read against denser ones in the nephelometer always show a lower nephelometric value. One method, therefore, of escaping from the difficulties which have been described is to have on hand enough of the test solution for several determinations. It is well also to have a series of standard phosphate solutions of graded concentrations, the range of which should extend from the lower to the upper

TABLE IV.

	P concentrations in solutions from which suspensions were made.	Ratio between densities of pairs of suspensions.	Nephelometric readings.	Ratio between nephelometric values of pairs of suspensions.
	per cent		*mm.*	
Suspensions precipitated at about 28°.	$\frac{0.00019}{0.00027}$	0.70	$\frac{30.0}{23.8}$	0.79
Suspensions precipitated at about 7°.	$\frac{0.00019}{0.00027}$	0.70	$\frac{30.0}{21.8}$	0.73

limit of the range within which the concentration of the test solution might lie. The test is first read against that standard which is guessed to be nearest to it; and, under ordinary circumstances, this preliminary reading should make it possible to pick out a standard for a second confirmatory reading, whose difference from the test will lie very close to the limits of accuracy attained in any ordinary chemical work.

It is necessary also, however, to test the reagent frequently by using it to read two moderately different standard solutions against each other. For if, as is commonly the case with old reagents and with reagents made up with strong hydrochloric acid, the differences in nephelometric value are strongly minimized, the experimenter may be deceived in the preliminary test into thinking that the standard and test solutions have nearly

equal concentrations, when this is not really the case at all. In my experience, it has been necessary to test the reagent in the manner described at least every other day.

It is obvious that when the determinations are carried out as described above, one is more likely to be deceived by a reagent that minimizes the nephelometric differences than by one that exaggerates them. But reagents which greatly exaggerate the nephelometric differences are likely to be very unstable. In my experience those reagents are likely to be most serviceable which give nephelometric readings about equal to what they should be theoretically. This condition is approximately attained for the Bloor procedure in summer (in the neighborhood of Washington) by using a 1:1 hydrochloric acid which contains 19 per cent HCl. When the temperature is lower, I have found it advantageous to use a 1:1 hydrochloric acid which contains from 17 to 18 per cent HCl.

My experience with the nephelometric method began with a period of some weeks which I spent in learning and practising it under Dr. Bloor's direction, and I take this opportunity of expressing my sincere thanks for his kindness and very essential help and advice.

NOTE ON THE PREPARATION OF GULONIC LACTONE.

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(Received for publication, August 25, 1918.)

In synthetic investigations in the sugar group, it is often necessary to prepare one sugar from another having a lower number of carbon atoms.

To attain this object, only one procedure has been found successful; namely, the cyanhydrin method of Kiliani and Fischer. In the practise of this synthesis, the sugar in question is brought into contact with about the theoretical amount of hydrocyanic acid in aqueous solution, a small amount of ammonium hydroxide is generally added as a catalytic agent, and the reaction is allowed to proceed for many hours or days. The resulting solution is then boiled with an excess of a barium hydroxide to effect the saponification of the two nitriles formed by the addition of the hydrocyanic acid to the sugar, and at the same time to expel the ammonia resulting from the saponification. The two epimeric acids resulting from this reaction are present in the solution as barium salts and it is then necessary to remove the base by means of sulfuric acid, after which the acids are isolated as lactones, salts, or other derivatives. The corresponding sugars are obtained by reduction of the lactones of the acids.

Although this method always produces good results, it is nevertheless possible in its application in certain instances at least, to introduce very marked improvements. Such an instance is for example in the preparation of gulonic lactone and, hence of gulose, from xylose.

The simplified method about to be described, makes possible the elimination of the operation of boiling with barium hydroxide; the saponification of the nitrile of gulonic acid being effected with a slight excess of sulfuric acid. The resulting ammonium sulfate

is allowed to remain in the solution and in no wise interferes with the crystallization of the lactone, which takes place on concentration of the reaction product.

It became evident from the experiment described below, as was shown by polarimetric readings, that in the preparation of gulonic lactone only about 6 hours are required for complete reaction between the xylose and hydrocyanic acid.

If the pure sugar was used for the synthesis, very little color developed during the reaction and the solutions were finally only light yellow. The yield of chemically pure, recrystallized gulonic lactone amounted to 55 per cent of the weight of the xylose employed.

There is no doubt that the method could be applied in the preparation of glucoheptonic lactone from glucose and, with proper modifications, in other instances. In case the lactones in question were soluble in alcohol, for example, the ammonia could be removed as the chloride by means of this solvent.

Preparation of Gulonic Lactone.—150 gm. of xylose were dissolved in 300 cc. of water and 30 gm. (slightly more than the equivalent amount) of hydrocyanic acid were added to the solution. Upon addition of a few drops of ammonium hydroxide, reaction began as was evident from the increase in temperature of the solution. A polariscopic reading taken after about 25 minutes showed a rotation in a 1 dm. tube of $+2.5°$; showing that even after that short time, the reaction was very far advanced. After four hours, during which time the temperature of the solution was not permitted to go above 35°, a second reading showed a rotation of $+1.2°$ and this value remained practically constant during the following 16 hours. At this stage 1 cc. of the solution, after boiling with a few drops of concentrated hydrochloric acid, gave a reduction of Fehling's solution, corresponding to 0.1200 gm. of Cu_2O, showing that 90 per cent of the reducing sugar had disappeared. For subsequent preparations, 6 hours were shown to be sufficient time for the addition of hydrocyanic acid to xylose.

Slightly over one equivalent (55 gm.) of sulfuric acid diluted with a small amount of water was added to the solution, which was then concentrated at once to a thick syrup. Crystallization began almost immediately, but the mass of syrup and crystals

was allowed to stand over night, after which the crystals were filtered off on silk with suction. The crude product was nearly pure white and the mother liquor only slightly colored. The presence of ammonium sulfate does not interfere with the isolation of the idonic acid which may be accomplished by the benzaldehyde method.[1]

The crude gulonic lactone may be recrystallized from water, in which case the mother liquor must be concentrated to recover the dissolved portion, or from 60 per cent alcohol. The yield of recrystallized material amounts to 55 to 60 per cent of the weight of the xylose used.

[1] Van Ekenstein, W. A., and Lobry de Bruyn, C. A., *Rec. trav. chim. Pay-Bas*, 1899, xviii, 305.

THE DETERMINATION OF CARBON DIOXIDE IN CARBONATES.

By DONALD D. VAN SLYKE.

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(Received for publication, September 16, 1918.)

The method described below was devised primarily for use in determination of the carbonate in bones which had been dried and pulverized, but not ashed. This material offered peculiar difficulties to analysis by the usual methods. The method adopted appears to be applicable to all carbonates, soluble or insoluble, in the absence of acids, such as hydrogen sulfide, that are highly volatile from water solution. The principle of rapid extraction of carbon dioxide from solution by means of reduced pressure, utilized in the author's method for determining bicarbonates in blood[1] has been combined with the familiar precipitation of carbonic acid by standard barium hydroxide solution and titration of the excess of hydroxide. Under the reduced pressure the transfer of carbon dioxide to the barium hydroxide solution can be completed in 3 minutes, and the fact that the entire process is carried out in a single closed vessel practically excludes error from loss of carbon dioxide.

The carbonate, either pulverized or in solution, is placed in the bottom of a tube 20 to 25 mm. in diameter which is placed in a 250 cc. suction flask (Fig. 1) containing an excess of 0.1 N barium hydroxide. When pulverized carbonate is weighed into the tube, care must be taken that the entire amount is placed at the bottom, as portions sticking to the walls may fail to be decomposed by the acid added later. Our practice has been to weigh the tube, slip into it a roll of glazed paper to protect the walls, pour in the substance, and reweigh the tube after withdrawing the paper. When the tube is in place (Fig. 1), the flask is evacuated to a pressure of 50 mm. or less, and the outlet is closed with the screw clamp. An excess of normal hydrochloric acid, usually about 5

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

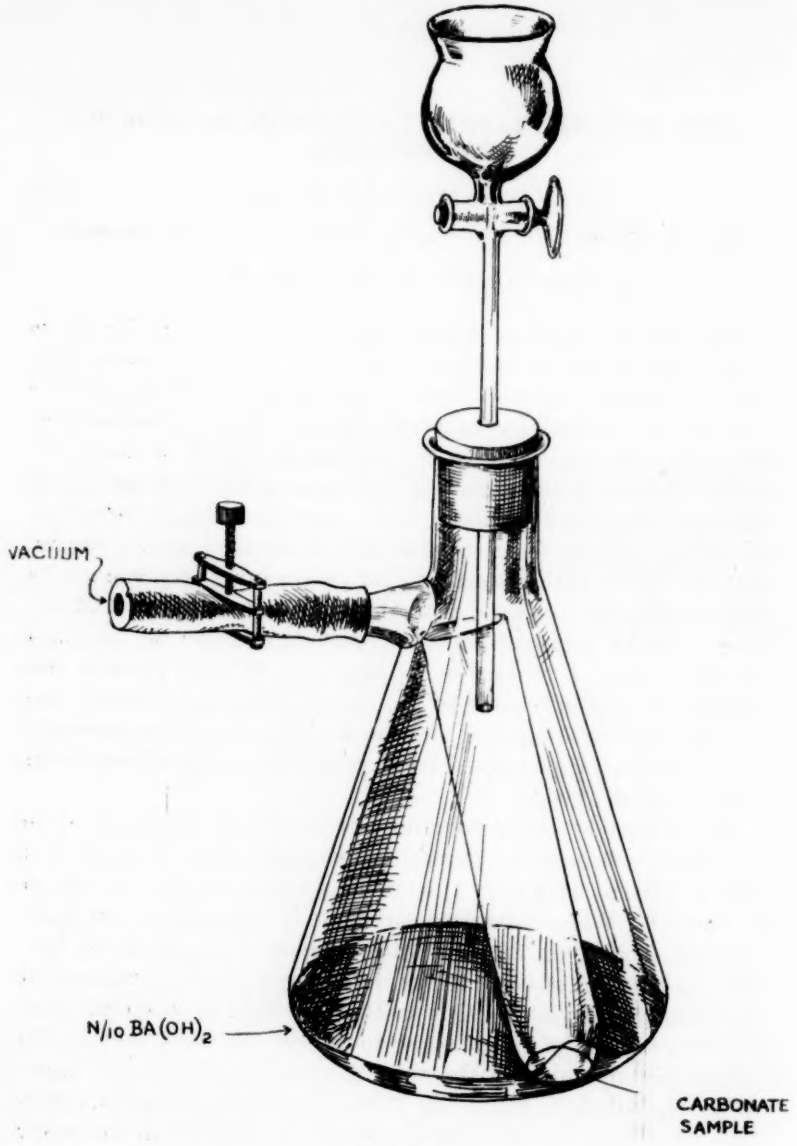

FIG. 1.

cc., is admitted slowly from the dropping funnel. Some of the solution is left above the stopper to assure its remaining air-tight. When the rapid evolution of carbon dioxide has ceased, the solutions are both agitated by a rotary motion for 3 minutes. In the analysis of sodium or calcium carbonate, this period has proved sufficient for complete transfer of carbon dioxide from the inner tube to the barium hydroxide solution. If the solution is not agitated, however, so that the surface of the barium hydroxide solution is continually renewed, the flask may stand for an hour without complete absorption of the carbon dioxide.

In the analysis of bones, which have been pulverized but not ashed, time must be allowed for the acid to penetrate into the particles, which are permeated with fat and protein. In this case it has been found necessary to allow at least 2 hours instead of 3 minutes for complete evolution and absorption of carbon dioxide. In routine analyses we have made a practice of allowing 5 hours. The solutions in the flask are stirred by rotation occasionally during this period, and for 3 minutes at the end of it.

After the reaction is completed the vacuum is released, and the barium carbonate is removed by filtration with a Gooch crucible, flask and crucible being rinsed with 3 portions of about 20 cc. each of water. The filtrate in the receiving suction flask is titrated against 0.1 N hydrochloric acid using phenolphthalein as indicator.

EXPERIMENTAL.

Sodium Carbonate.—A sodium carbonate solution was prepared containing 10 gm. of Merck's anhydrous Na_2CO_3 per 100 cc. Titration with methyl orange indicated that the solution was of the calculated concentration. 1 cc. portions, measured to within 0.001 cc. with an Ostwald pipette, required 18.93 and 18.87 cc. of 0.1 N HCl, the calculated amount being 18.85.

Calcium Carbonate.—The calcium carbonate used was a Baker and Adamson's C. P. precipitated. To check its purity varying amounts were dissolved in excess 0.1 N hydrochloric acid, the carbon dioxide was expelled by boiling, and the excess acid titrated back with 0.1 N barium hydroxide, using alizarin sulfonate as indicator. The 0.1 N acid had been prepared by the method of Hulett and Bonner.[2]

[2] Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, xxxi, 390.

TABLE I.

$CaCO_3$	0.1 N HCl	0.1 N $Ba(OH)_2$	0.1 N HCl neutralized by $CaCO_3$.		$CaCO_3$ in preparation used.
			Observed.	Calculated.	
gm.	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*
0.4424	100.00	12.20	87.80	88.48	99.3
0.2060	50.00	9.29	40.71	41.20	98.8
0.2105	50.00	8.36	41.64	42.10	98.9
Average.....					99.1

In accordance with the above results, the weights of calcium carbonate taken in the following analyses were corrected by multiplication by the factor 0.991, and the weights given in Table II are so corrected.

TABLE II.

Determination of Carbon Dioxide in Sodium Carbonate Solution and in Pulverized Calcium Carbonate.

Na_2CO_3 (in 10 per cent solution).	$CaCO_3$	0.1 N $Ba(OH)_2$ in flask.	0.1 N HCl to titrate excess $Ba(OH)_2$.	0.1 N $Ba(OH)_2$ precipitated by CO_2.	CO_2 found.	CO_2 present.
gm.	*gm.*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*
0.100	—	29.00	10.20	18.80	0.0414	0.0415
0.100	—	29.00	10.15	18.85	0.0415	0.0415
0.100	—	29.00	10.20	18.80	0.0414	0.0415
—	0.0730	20.28	5.55	14.73	0.0324	0.0321
—	0.0909	20.28	2.00	18.25	0.0402	0.0400
—	0.1652	40.56	7.50	33.06	0.0727	0.0726
—	0.1709	40.56	6.20	34.36	0.0756	0.0752
—	0.1764	40.56	5.45	35.11	0.0773	0.0778

The accompanying paper by Dr. Goto gives the results obtained in bone analyses.

MINERAL METABOLISM IN EXPERIMENTAL ACIDOSIS.

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(Received for publication, September 16, 1918.)

No detailed study of the mineral changes in the soft tissues and skeleton respectively, caused by either clinical acidosis or prolonged acid feeding, has been published so far as we have been able to ascertain. We have only the observation which Gerhardt and Schlesinger (1899) report to have been made by von Frerich, that the skeletons of diabetics dying in coma appear to be atrophied. Quantitative work on the subject appears to have been limited chiefly to a study of excretion.

Knowledge derived from direct analysis of the body itself has been limited to the blood. Walter (1877) showed that entrance of acids into the circulation causes an immediate reduction of the blood bicarbonate, and Palmer and Van Slyke (1917) have found that the bicarbonate concentrations of the body fluids in general parallel those of the blood plasma, so that depletion of bicarbonate in the blood plasma indicates a depletion of the bicarbonate stores throughout the body. When such a bicarbonate deficit continues, some depletion of the other alkali reserves might also be expected, and the literature contains a considerable amount of metabolic work which indicates that it occurs.

Ash analyses indicate that the main reserves of alkali aside from the bicarbonates are the calcium carbonate and the much greater amount of calcium phosphate in the bones, and the potassium and sodium phosphates of the tissues; *i.e.*, the alkali reserves aside from the carbonates are chiefly phosphates. It would therefore be expected that if the reserves of either the skeleton or soft tissues are drawn upon to a considerable extent, an increased excretion of phosphates would result, chiefly sodium and potassium phosphates if the source were the soft tissues, calcium

phosphate if it were the bones. Increased phosphate excretion in the urine has in fact been reported in diabetic ketonuria and in experimental hydrochloric acid feeding in man and in animals.

In diabetic ketonuria, however, it is uncertain whether the increased phosphoric acid excretion is due to loss of alkaline phosphates from the body, or merely to combustion of increased amounts of food and body proteins. Gerhardt and Schlesinger (1899) as evidence that there is an excretion of P_2O_5 in excess of that accountable for by protein catabolism, report a $\frac{P_2O_5}{N}$ ratio of $\frac{18\text{–}20}{100}$ in the urines of two diabetics with heavy ketonuria. The normal ratio under similar conditions was estimated at $\frac{12.5}{100}$. Feeding sodium bicarbonate to the diabetics reduced it to $\frac{14\text{–}16}{100}$. Rumpf (1898) and Mandel and Lusk (1904) report in a fasting patient with severe diabetes $\frac{P_2O_5}{N}$ ratios similar to those of Gerhardt and Schlesinger. Folin and Shaffer (1902), however, found similar ratios, $\frac{18\text{–}20}{100}$, common in normal adults. Consequently, as stated above, the data at hand do not tell us whether or not the alkaline phosphates of the body are depleted in diabetic acidosis.

Increased excretion of calcium and magnesium in the urine has been reported in diabetic acidosis by Gerhardt and Schlesinger (1899) and by Tenbaum (1896). Lack of complete intake and outgo data, however, make it impossible to state whether the figures indicate a real loss of bone substance.

The metabolic experiments with acid feeding in the literature include observations more comprehensive than were made in the above cited studies on diabetics. Steenbock, Nelson, and Hart (1914) found that feeding hydrochloric acid to a calf increased the urinary phosphoric acid, but that even large doses showed no tendency to produce a negative phosphate balance. When the maximum dose of 400 cc. of 1 N HCl per day was given, a negative calcium balance was produced. The authors conclude that the acid attacked only the calcium carbonate, not the calcium phosphate of the bones.

Fitz, Alsberg, and Henderson (1907) observed in the urine of rabbits, which received dilute hydrochloric acid daily, a marked increase of phosphoric acid excretion, followed by a decrease, presumably when the readily available phosphates were exhausted. In some cases the excretion rose again before death.

Givens and Mendel (1917) examined the balance of nitrogen, calcium, magnesium, and phosphorus after the administration of base and acid in the dog. The phosphorus balance showed no significant effect and was always positive, though extremely variable quantitatively. The figures for both the urinary and the fecal phosphorus were so variable that neither could be used as an index of any influence exerted by sodium bicarbonate administration. Stehle (1917) states that the administration of hydrochloric acid by mouth to the dog causes an increased excretion of calcium and magnesium as well as of sodium and potassium.

Except for the results of Givens and Mendel (1917, 1918) with dogs, the above literature indicates that sufficient acid feeding produces in various animals an increased excretion of phosphate in the urine. The results of Fitz, Alsberg, and Henderson (1907), who followed the daily P_2O_5 excretion of rabbits fed with HCl for weeks until death resulted, are particularly significant.

In our experiments the chief object has been to ascertain the effect of acid feeding on the mineral composition of the bones and muscles respectively; but we have followed the phosphate excretion in the urines of our rabbits in order to make certain that the animals were actually responding with increased phosphate excretion in the manner to be expected from the experiments of Fitz, Alsberg, and Henderson. The results were uniformly confirmatory.

There are however, two possible explanations of the increased phosphate excretion in the urine caused by acid feeding. The increased urinary phosphate may be drawn from the reserves of the body to neutralize the acid, or it may be due merely to increased absorption of calcium and magnesium phosphates of the food transformed in greater amount into soluble forms by the ingested acid in the alimentary tract. Würtz (1912) it is true failed to find that feeding hydrochloric acid to rabbits had any effect on their output of P_2O_5 in either urine or feces. The

amounts of acid which he fed were, however, only about one-seventh as great as in the experiments of Fitz, Alsberg, and Henderson (1907), and for this reason his negative results are inconclusive. In order to ascertain whether the amounts of acid fed in our experiments influence the distribution of excreted phosphate between feces and urine, we have in some cases determined the excretion in both. The acid feeding showed no definite influence on the distribution. The increased phosphate excretion in the urine was not accompanied by a decrease in the feces, and consequently it appears that the urinary increase in our experiments indicates a genuine negative balance in phosphoric acid—a conclusion which is confirmed by the tissue analyses. These results lend added force to the conclusions of Fitz, Alsberg, and Henderson (1907), that their data indicated a utilization of body phosphates as alkaline reserve to neutralize invading acids and turn them into excretable salts.

EXPERIMENTAL.

I. Effect of Acid Feeding on Phosphate Excretion in Urine and Feces.

As experimental animals, rabbits were chosen in preference to dogs. The ammonia-forming power of dogs enables them to neutralize great amounts of acid without drawing upon the body reserves of mineral alkali. Rabbits can form ammonia much less rapidly (Walter, 1877), and can therefore be expected to neutralize unusual amounts of strong acid only by means of the reserves of alkali in the body fluids, tissues, and skeleton.

The rabbits were kept in metabolism cages and were offered daily 65 gm. of bread and 300 gm. of cabbage. They did not usually consume all that was given, as shown by Table VII. The acid was given in 0.25 N concentration by stomach tube, the amounts varying from 25 to 75 cc. daily. The conditions approximate those of Fitz, Alsberg, and Henderson (1907).

The urine was collected daily at 9 a.m. from Monday to Saturday, and kept in a refrigerator; the urine for the 5 week days was mixed and the total phosphoric acid estimated by titration with uranium nitrate.

Feces were collected daily at 3 p.m. before giving food, and the corresponding amounts of food and feces noted. Feces for 5 days, from Monday to Saturday, were mixed in one dish and ashed. The ash was dissolved by boiling with hydrochloric acid, and the solution was filtered into a 250 cc. volumetric flask and diluted to the mark. Phosphoric acid in 25 cc. portions was precipitated as molybdate and weighed as $Mg_2P_2O_7$.

Results.—The rabbits showed a poor resistance to hydrochloric acid, most of them dying within 1 or 2 weeks. Among the many

TABLE I.

Phosphorus Excretion in Urine of Normal Rabbits.

Date of experiment.		PO_4 in urine per day.			
		Rabbit VIII (body wt. 1,500 gm.).	Rabbit X (body wt. 1,550 gm.).	Rabbit XV (body wt. 1,500 gm.).	Rabbit XVI (body wt. 1,400 gm.)
wk.	*days*	*gm.*	*gm.*	*gm.*	*gm.*
1st	1–6	0.039	0.057	0.037	0.032
2nd	8–13	0.035	0.044	0.066	0.032
3rd	15–20	0.043	0.074 Mixed with feces.	0.017	0.086 Mixed with feces.
4th	22–27	0.033	0.041	0.024	0.020
5th	30–34 (4 days)				0.063
Average ...		0.038	0.047 (1st, 2nd, 4th weeks.)	0.036	0.037 (1st, 2nd, 4th, 5th weeks).

rabbits, which were given 50 cc. of 0.25 N HCl daily from the beginning of the experiment, only Rabbit III lived more than 2 weeks. As the object of the experiment was to see the effect of continued acidosis, possibly similar to diabetic acidosis, upon the metabolic changes of the body tissues, it was intended to keep the rabbits at least 4 weeks. Therefore, 25 cc. doses of 0.25 N HCl were given to several of the rabbits. Of these, Rabbit V lived a long time and received increased doses of acid. Rabbits XI and XII received 30 cc. of 0.25 N HCl from the 2nd week.

TABLE II.

Excretion of PO_4 in Urine of Rabbits Fed with HCl.

Date of experiment.		Acid-fed Rabbit III.			Acid-fed Rabbit IX.			Acid-fed Rabbit XX.			Acid-fed Rabbit XXI.		
		PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.
wk.	*days*	*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*	
1st	1–6	0.075	1,500	50 cc. 0.25 N HCl daily.	0.096	1,500	25 cc. 0.25 N HCl daily.	0.110	1,850	30 cc. 0.25 N HCl daily.	0.091	1,400	30 cc. 0.25 N HCl daily.
2nd	8–13	0.135	1,450	50 cc. 0.25 N HCl daily.	0.088	1,400	25 cc. 0.25 N HCl daily.	0.120	1,550	30 cc. 0.25 N HCl daily.			Died 7th day.
			1,310	Died 15th day.			Died 15th day.			Died 15th day.			

TABLE III.

Excretion of PO_4 in Urine of Rabbits Fed with HCl.

Date of experiment.		Acid-fed Rabbit V.			Acid-fed Rabbit XII.			Acid-fed Rabbit XI.			Acid-fed Rabbit VII.				
		PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	PO_4 in urine per day.	Wt. of rabbit.	Remarks.	Date of experiment.		PO_4 in urine per day.	Wt. of rabbit.	Remarks.
wk.	*days*	*gm.*	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*		*wk.*	*days*	*gm.*	*gm.*	
1st	1–6	0.122	1,450	25 cc. 0.25 N HCl daily.	0.037	1,650	25 cc. 0.25 N HCl daily.	0.078	1,300	25 cc. 0.25 N HCl daily.	1st	1– 3	0.099	1,500	25 cc. 0.25 N HCl daily.
2nd	8–13	0.058	1,300	25 cc. 0.25 N HCl daily.	0.086	1,650	30 cc. 0.25 N HCl daily.	0.051	1,250	30 cc. 0.25 N HCl daily.	2nd	5–10	0.082	1,450	25 cc. 0.25 N HCl daily.
3rd	15–20	0.039	1,250	25 cc. 0.25 N HCl daily.	0.054	1,600	30 cc. 0.25 N HCl daily.	0.036	1,250	30 cc. 0.25 N HCl daily.	3rd	12–17	0.087	1,200	25 cc. 0.25 N HCl daily. Died 17th day.
4th	22–27	0.080	1,250	35 cc. 0.25 N HCl daily.			Died 22nd day.	0.012	1,150	30 cc. 0.25 N HCl daily.					
5th	29–34	0.056	1,250	50 cc. 0.25 N HCl daily.				0.017	1,150	30 cc. 0.25 N HCl daily.					
6th	36–38	0.088	1,250	75 cc. 0.25 N HCl daily. Died 38th day.						Died 35th day.					

Rabbits XX and XXI received 30 cc. of 0.25 N HCl from the beginning of the experiment, and lived less than 3 weeks, but were examined to ascertain the effect of the shorter period of acid feeding.

Four normal controls of almost similar weight showed an average daily PO_4 excretion of 0.036 to 0.047 gm. (Table I).

TABLE IV.

Phosphorus in Feces of Normal Rabbits.

Date.		PO_4 per day.	
		Rabbit XV.	Rabbit XVI.
wk.	*days*	*mg.*	*mg.*
3rd	15–20	12.5	38.6
4th	22–27	40.7	87.5
5th	30–34		65.0

TABLE V.

Phosphorus in Feces of Acid-Fed Rabbits.

Date of experiment.		PO_4 per day.			
		Rabbit XI.	Rabbit XII.	Rabbit XX.	Rabbit XXI.
wk.	*days*	*mg.*	*mg.*	*mg.*	*mg.*
1st	1–6			39.6	56.9
2nd	8–13			49.5	
3rd	15–20	120	153		
4th	22–27	112			
5th	30–34	26			

Of the experimental rabbits, Nos. III, V, VII, IX, XI, XX, and XXI excreted more phosphate even in the 1st week than the controls (Tables II and III). Rabbit XII did not show the increased excretion in the 1st week, but did show an excessive excretion in the 2nd and 3rd weeks. Rabbit XI showed a normal excretion in the 3rd week, followed by diminished excretion in the 4th and 5th weeks. Rabbit V also showed a normal

TABLE VI.

Diet of Normal Rabbits in Which Phosphoric Acid of Feces Was Determined.

Date of experiment.		Rabbit XV.		Rabbit XVI.	
		Bread.	Cabbage.	Bread.	Cabbage.
wk.	*days*	*gm.*	*gm.*	*gm.*	*gm.*
3rd	15–16	65	300	65	300
	16–17	65	285	47	235
	17–18	50	187	52	200
	18–19	27	121	57	270
	19–20	37	130	52	202
4th	22–23	45	243	48	233
	23–24	58	223	58	300
	24–25	47	193	50	300
	25–26	61	220	54	222
	26–27	—	—	—	—
5th	30–31	—	—	58	300
	31–32	—	—	59	300
	32–33	—	—	43	172
	33–34	—	—	46	154

TABLE VII.

Diet of Acid-Fed Rabbits in Which Phosphoric Acid of Feces Was Determined.

Date of experiment.		Rabbit XI.		Rabbit XII.		Date of experiment.		Rabbit XX.		Rabbit XXI.	
		Bread.	Cabbage.	Bread.	Cabbage.			Bread.	Cabbage.	Bread.	Cabbage.
wk.	*days*	*gm.*	*gm.*	*gm.*	*gm.*	*wk.*	*days*	*gm.*	*gm.*	*gm.*	*gm.*
3rd	15–16	65	275	65	300	1st	1–2	—	300	—	300
	16–17	65	229	42	239		2–3	—	300	—	245
	17–18	53	166	56	200		3–4	40	200	—	157
	18–19	48	193	41	268		4–5	54	300	46	126
	19–20	49	148	38	171		5–6	38	300	31	98
4th	22–23	38	281			2nd	8–9	16	226		
	23–24	47	204				9–10	45	293		
	24–25	12	107				10–11	22	178		
	25–26	19	168				11–12	17	133		
	26–27	—	—				12–13	—	—		
5th	30–31	44	171								
	31–32	42	139								
	32–33	31	143								
	33–34	20	135								

phosphate excretion in the 3rd week, but as he received gradually increased doses of acid after that period, the results are not comparable with those of Rabbit XI. When the amount of ingested hydrochloric acid rose, a corresponding increase of phosphoric acid excretion occurred.

The PO_4 in the feces of the control rabbits was 12.5 to 87.5 mg. per day (Table IV), in the acid-fed rabbits 26 to 153 mg. (Table V). It is evident that the acid feeding caused no decrease in fecal phosphorus, the change being, if anything, in the opposite direction.

II. The Influence of Acid Feeding in the Rabbit on the Bicarbonate Content of the Plasma.

Walter (1877), who made the first experiments in acid intoxication with dogs and rabbits, found great decrease in the carbon dioxide content of the blood. The results of later experimenters agree with Walter's. The effects of HCl administration in the dog upon the CO_2 content of the plasma, estimated by the Van Slyke method, were recently reported by the writer (1917).

25 cc. of 0.25 N HCl were administered by stomach tube to several normal rabbits daily at 2 p.m. Blood was drawn by heart puncture before the acid feeding, and the CO_2 content of the plasma estimated by the Van Slyke method (Table VIII). The rabbits died in about 1 week after the acid administration, since in their weakened condition they could not stand the heart puncture. Bicarbonate CO_2 in the plasma of the controls was 44 to 64 per cent. In the 1st week of the acid administration (3rd day) several rabbits showed a lowered CO_2 in the plasma. These rabbits did not live to the 2nd week. Rabbit XXVIII showed no acidosis in the 1st, but a marked acidosis in the 2nd week. Rabbit XXVII, which was not bled in the 2nd week but which received acid every day, also showed a marked acidosis in the 3rd week. Both rabbits died after heart puncture. Rabbit XI, used in one of the previous experiments, showed 34.9 per cent CO_2 content in the plasma in the 4th week (April 30).

TABLE VIII.

Carbon Dioxide Content of Plasma.

	Date.	Rabbit XXIV.	Rabbit XXV.	Rabbit XXVI.	Rabbit XXVII.	Rabbit XXVIII.	Rabbit XXIX.	Remarks.
		vol. per cent	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	
Normal condition.	May 21	44.0	44.0	44.0	57.2	60.0	53.4	
	" 24	54.4	66.0	63.9	51.5	57.3	63.0	
1st week of acid feeding.	May 29	31.2 Died May 30.	45.6	36.9 Died May 31.	51.1	63.8	43.6 Died May 31.	From May 27. 25 cc. 0.25 N HCl given daily.
2nd week of acid feeding.	June 5		Died June 2.			33.6 Died June 5.		
3rd week of acid feeding.	June 12				31.2 Died June 12.			

III. Mineral Changes in Muscles and Bones.

After the deaths of the rabbits which had been used for the phosphate excretion experiments reported above, the muscles and bones were separated as completely as possible. The total muscles (except the heart) were weighed, ground in a food chopper, weighed again, and kept in a refrigerator. From them phosphorus, potassium, and sodium were determined. The bones from the entire body, including the bone marrow, were weighed, dried on a water bath for 4 or 5 days, ground in a hand mill, weighed again, and kept in a bottle. In the bone powder, phosphoric acid, calcium, carbonic acid, and fat were estimated.

Age effects a change in the mineral constituents of the bones of rabbits (Weiske, 1872, Wildt, 1872), but the changes are mini-

mal in rabbits from 1 to 3 or 4 years of age. Adult animals from 1 to 2 years of about the same weight were selected for the present research.

Methods of Analysis.

Phosphoric Acid in Muscle Ash.—50 gm. of fresh muscle were weighed into a platinum dish and ashed, first over a flame and finally in a muffle furnace, using full red heat only after all organic matter was entirely turned to charcoal. To the residue 15 cc. of water and 3 to 5 cc. of concentrated hydrochloric acid were added, and the ash was dissolved with the aid of heat. The solution was washed into a 200 cc. flask, diluted to the mark, and filtered into an Erlenmeyer flask through a dry folded filter paper to remove particles of carbon. 50 cc. portions of this solution were taken for phosphate determination. The phosphate was precipitated as molybdate and weighed as $Mg_2P_2O_7$.

Potassium and Sodium in Muscle.—After ashing the muscle and dissolving the residue in the same manner, 5 cc. of 10 per cent $BaCl_2$ were added and followed by 10 per cent $Ca(OH)_2$ suspension till the solution was alkaline to litmus. The mixture was diluted to 150 cc. and filtered through a folded filter paper to remove phosphates and sulfates. To 100 cc. of the filtrate 20 cc. of saturated ammonium oxalate were added, followed by 10 per cent ammonium carbonate until no further precipitate was obtainable. The solution was diluted to 200 cc. and filtered through a folded filter paper to remove calcium and barium. 50 cc. of the filtrate were placed in a weighed silica dish with an excess of concentrated hydrochloric acid and evaporated to dryness on a water bath. The residual KCl and NaCl were ignited and weighed. The chlorides were dissolved in a small amount of water, 0.5 cc. of perchloric acid was added, and the solution evaporated to dryness. After addition of 3 or 4 cc. of water and five drops of perchloric acid, the solution was evaporated to dryness again. The residue was stirred up with 10 cc. of 97 per cent alcohol containing 0.2 per cent of perchloric acid, allowed to stand at least 20 minutes, and then transferred to a Gooch crucible, in which it was washed several times with small portions of the same alcoholic solution containing 0.2 per cent of perchloric acid. The $KClO_4$ was dried at 110°C. and weighed.

Phosphoric Acid Determination in Bones.—10 gm. of dried bone powder were ashed in a platinum dish as above. The ash was transferred to a 200 or 300 cc. beaker, with 50 to 100 cc. of water. The latter was boiled and hydrochloric acid added until all solid matter except carbon was dissolved. The solution was filtered into a 250 cc. flask. The phosphate in 20 cc. was precipitated as molybdate and weighed as $Mg_2P_2O_7$.

Calcium Determination in Bones.—To 10 cc. of the bone ash solution, prepared as above, 5 cc. of 10 per cent sulfuric acid and 60 cc. of 95 per cent alcohol were added. The solution was let stand over night. The $CaSO_4$ precipitate was filtered into an alundum crucible, washed with 70 per cent alcohol, and dried at 100°. The alundum crucible was then set inside a porcelain one, which was heated to a dull red for about 30 minutes.

Carbonic Acid Determination in Bones.—Van Slyke's new carbonate method was used (1918), as described in the preceding article. 1 gm. samples were used of the dried bone powder.

Determination of Fat in Bone.—1 gm. of dried bone powder was extracted in a Soxhlet extractor with 150 cc. of absolute ether for 24 hours. The ether was driven off on a water bath. 50 cc. of petroleum ether were added and let stand over night on the residue. The solution was filtered through fat-free cotton, the petroleum ether drawn off, and the residue weighed as fat.

Results.

I. Muscles.

1. Weight of Muscles.—The weight of the total muscles per kilo of body weight in four control rabbits was practically constant, varying only from 318 to 355 gm. (Table IX). In the experimental rabbits, the ratio of weight of muscles to body weight was calculated on the basis both of final body weight and of body weight before acid feeding was begun, since the animals lost weight during the period of acid feeding. The weight of muscles per kilo of initial body weight, except for Rabbits XII and XXI, was lower in the acid-fed rabbits than in any of the controls, indicating a loss of muscle tissue during acid feeding (Table X).

The weight of muscles per kilo of final body weight was lower than normal in Rabbits V, VII, IX, XI, and XX which had received a long continued acid feeding (13 to 38 days). The ratio in Rabbits XXI and XII was almost normal, in spite of the fact that they had received acid for 2 weeks or more. These

TABLE IX.

Weight of Muscles in Normal Rabbits.

Rabbit No.	Body wt.	Weight of muscles.		
		Before maceration.	After maceration.	
				Per kg. body wt.
	gm.	*gm.*	*gm.*	*gm.*
VIII	1,500	530.6	493.3	355
X	1,500	521.6	477.1	318
XV	1,500	544.0	523.8	350
XVI	1,400	476.8	449.0	321
Average.....				336

TABLE X.

Weight of Muscles in Acid-Fed Rabbits.

Rabbit No.	Duration of experiment.	Body weight.		Weight of muscles.		Weight of muscles.	
		Initial.	Final.	Before maceration.	After maceration.	Per kg. initial body wt.	Per kg. final body wt.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
III	15	1,500	1,310	430.5	415.0	277	320
V	38	1,450	1,250	335.2	319.5	220	255
VII	17	1,500	1,200	347.1	331.8	222	277
IX	13	1,500	1,400	442.1	424.0	283	303
XI	35	1,240	1,150	272.0	262.8	212	229
XII	22	1,650	1,600	579.0	561.0	340	350
XX	14	1,850	1,500	486.0	472.0	255	314
XXI	7	1,400	1,340	482.0	463.0	331	346

results show that such loss of weight as occurred fell more heavily on the muscles than on the body as a whole.

2. Change in Phosphorus Content of Muscles.—The control rabbits showed almost constant results, the percentage of PO_4 in the fresh muscles being 0.759 to 0.883 gm., and the total PO_4 per kilo of body weight being 2.6 to 2.8 gm. The percentage

of PO_4 in the muscles of acid-fed rabbits was in some cases normal, but in four of the eight animals it was lower than the lowest normal. The ratio of total PO_4 per kilo of initial body weight was remarkably lower than normal in all the rabbits which had received acid except Rabbit III. From the results it is evident that the total phosphorus in the muscles of rabbits is reduced by acid feeding, and that on the average, the reduction includes the percentage of phosphorus in the muscles as well as the amount in the total musculature.

3. Changes in Sodium Content of the Muscles.—The sodium content in the muscles of the normal animals showed an average of 0.0973 per cent, the extremes being 0.078 to 0.122. The total

TABLE XI.

Analysis of Muscle of Normal Rabbits.

Rabbit No.	PO_4			Na			K		
	PO_4 in muscles.	Total PO_4 in all muscles.	Total PO_4 per kilo body wt.	Na in muscles.	Total Na in all muscles.	Total Na per kilo body wt.	K in muscles.	Total K in all muscles.	Total K per kilo body wt.
	per cent	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*
VIII	0.777	3.84	2.6	0.0988	0.487	0.33	0.301	1.49	0.99
X	0.883	4.21	2.8	0.122	0.582	0.39	0.252	1.20	0.80
XV	0.759	3.97	2.7	0.0903	0.473	0.32	0.32	1.68	1.12
XVI	0.796	3.58	2.6	0.078	0.351	0.25	0.385	1.73	1.24
Average...	0.804		2.64	0.0973		0.32	0.315		1.04

sodium per kilo of body weight was 0.25 to 0.39 gm., the average being 0.32 gm. for the four controls. A moderately high percentage of sodium is seen in the acid-fed rabbits, Nos. III, VII, and XXI. The ratio of the total sodium, both per kilo of initial body weight and per kilo of final body weight was lower than in the lowest control in Rabbits V, XI, and XX, which had received long courses of acid feeding.

4. Changes in Potassium Content of the Muscles.—In the normal rabbits the percentage of potassium in the muscles was 0.252 to 0.385, and the total potassium per kilo of body weight was 0.8 to 1.24 gm., the average being 1.04 gm. The percentage of potassium in the muscles of acid-fed rabbits, Nos. V, XII, and XXI, was lower than the lowest normal. The ratio of total potas-

TABLE XII.

Analyses of Muscles of Acid-Fed Rabbits.

Rabbit No.	Duration of experiment.	No. of days of acid feeding.	Total volume of 0.25 N HCl fed.	PO_4				Na				K			
				PO_4 in muscles.	Total PO_4 in all muscles.	Total PO_4 in muscles.		Na in muscles.	Total Na in all muscles.	Total Na in muscles.		K in muscles.	Total K in all muscles.	Total K in muscles.	
						Per kg. initial body wt.	Per kg. final body wt.			Per kg. initial body wt.	Per kg. final body wt.			Per kg. initial body wt.	Per kg. final body wt.
	days		*cc.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*
V	38	33	1,185	0.752	2.41	1.7	1.9	0.086	0.275	0.19	0.22	0.163	0.52	0.36	0.42
XI	35	29	810	0.827	2.17	1.8	1.9	0.084	0.221	0.18	0.19	0.251	0.66	0.58	0.59
III	15	12	600	0.933	3.87	2.6	2.95	0.132	0.584	0.37	0.42	0.288	1.195	0.80	0.91
XII	22	18	510	0.689	3.86	2.4	2.4	0.087	0.488	0.30	0.31	0.219	1.23	0.75	0.77
VII	17	15	375	0.595	1.99	1.3	1.7	0.141	0.467	0.31	0.39	0.268	0.896	0.59	0.74
XX	14	12	360	0.849	4.01	2.2	2.7	0.067	0.316	0.17	0.21	0.302	1.435	0.77	0.95
IX	13	12	300	0.733	3.11	2.1	2.2	0.0973	0.413	0.28	0.30	0.254	1.08	0.72	0.77
XXI	7	6	180	0.604	2.80	2.0	2.1	0.132	0.611	0.44	0.46	0.222	1.03	0.74	0.77

sium per kilo of initial body weight of all the acid-fed rabbits except No. III was lower than the lowest normal. The ratio of total potassium per kilo of final body weight in all the experimental animals, except Nos. III and XX, was lower than normal. It appears that potassium is considerably decreased in the muscles by experimental acidosis.

II. Bones.

1. Changes in Weight of Dried Skeleton.—The weight of dried bone per kilo of body weight was 54 to 65 gm. in the normal rabbits and averaged 59 gm. (Table XIII). The weight of bone per kilo of initial body weight was lowered to 40 to 54 gm. in the acid-fed rabbits averaging 50 gm. (Table XIV). The

TABLE XIII.

Weight of Skeleton in Normal Rabbits.

Rabbit No.	Wet.	After drying.	After crushing.	Per kg. body wt.
	gm.	*gm.*	*gm.*	*gm.*
VIII	140.5	86.2	80.8	54
X	158.9	94.4	83.0	55
XV	169.0	99.4	97.4	65
XVI	130.5	87.6	87.4	62
Average.....				59

ratio of the weight of bone per kilo of final body weight was normal in the acid-fed rabbits, except in Rabbit V which had endured a long continued acid feeding. These results indicate that the dry weight of the skeleton decreases parallel with the weight of the total body during acid feeding. As shown below, about half the bone loss is attributable to decrease in the bone fat.

2. Changes in Skeletal Phosphate.—The PO_4 percentage in the dried bones of normal rabbits was 20.8 to 23.3 and the total PO_4 per kilo of body weight was 11.2 to 14.3 gm.

All of the acid-fed rabbits showed a percentage of PO_4 in the bones rather higher than normal because of a decreased fat content, rather than an increase of phosphate, as will be indicated later. The ratio of the total PO_4 per kilo of initial body weight

was lower than normal only in Rabbit V of the acid-fed rabbits. Rabbit V showed a smaller amount of all bone substances. Whether this was due to the exceptional amount of acid given or

TABLE XIV.

Weight of Skeleton in Acid-Fed Rabbits.

Rabbit No.	Duration of experiment.	Weight of bone.				
		Wet.	After drying.	After crushing.	Per kg.	
					Initial body wt.	Final body wt.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
III	15	109		71.8	48	55
V	38	119	61	58.5	40	47
VII	17	134		68.5	46	57
IX	13	148		81.1	54	58
XI	35	129	65.2	65.2	53	57
XII	22	166	90.4	88.4	54	55
XX	14	158	95	93.6	51	62
XXI	7	158	75.6	72.4	52	54

TABLE XV.

Analysis of Bone of Normal Rabbits.

Rabbit No.	PO_4			Ca			CO_2			Fat.		
	PO_4 in dry bone.	PO_4 in total skeleton.	Total PO_4 per kg. body wt.	Ca in dry bone.	Ca in total skeleton.	Total Ca per kg. body wt.	CO_2 in dry bone.	CO_2 in total skeleton.	Total CO_2 per kg. body wt.	Fat in dry bone.	Fat in total skeleton.	Total fat per kg. body wt.
	per cent	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*
VIII	20.8	16.8	11.2	13.1	10.6	7.6	5.1	4.1	2.8	19.59	15.8	10.5
X	21.1	17.5	11.7	14.1	11.7	7.8	5.2	4.3	2.9	14.5	12.0	8.0
XV	21.7	21.0	14.0	14.7	14.3	9.5	4.8	4.7	3.1	18.21	17.7	11.8
XVI	23.3	20.3	14.3	15.2	13.3	9.5	4.9	4.3	3.1	16.22	14.1	10.1
Average.	21.7		12.8	14.3		8.6	5.0		2.95	17.13		10.1

to a chance individual variation from the normal, one cannot say. The bones of the other rabbits indicate no significant effect of the acid feeding on the phosphate content of the skeleton.

TABLE XVI.

Analyses of Bones of Acid-Fed Rabbits.

Rabbit No.	Duration of experiment.	No. of days of acid feeding.	Total volume of 0.25 N HCl fed.	PO_4				Ca				CO_2				Fat.			
				PO_4 in dry bone.	PO_4 in total skeleton.	PO_4 per kg. initial body wt.	PO_4 per kg. final body wt.	Ca in dry bone.	Ca in total skeleton.	Ca per kg. initial body wt.	Ca per kg. final body wt.	CO_2 content of dry bone.	CO_2 in total skeleton.	CO_2 per kg. initial body wt.	CO_2 per kg. final body wt.	Fat in dry bone.	Fat in total skeleton.	Bone fat per kg. initial body wt.	Bone fat per kg. final body wt.
	days		*cc.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*
V	38	33	1,185	24.2	14.2	9.8	11.3	15.25	8.9	6.2	7.1	5.1	2.98	2.1	2.4	8.97	5.3	3.6	4.2
XI	35	29	810	25.7	16.7	13.5	14.6	17.1	11.2	9.0	9.7	4.7	3.06	2.5	2.7	3.53	2.3	1.9	2.0
III	15	12	600	24.9	17.9	11.9	13.6	16.8	12.1	8.0	9.2	5.1	3.66	2.4	2.8	9.88	7.1	4.7	5.4
XII	22	18	510	21.4	19.8	12.0	12.4	14.1	12.5	7.6	7.8	4.6	4.07	2.5	2.5	14.03	12.4	7.5	7.8
VII	17	15	375	27.2	18.6	12.4	15.5	16.85	11.6	7.7	9.7	5.0	3.42	2.3	2.9	7.02	4.8	3.2	4.0
XX	14	12	360	25.8	24.1	13.1	16.0	17.9	16.8	9.1	11.2	5.6	5.27	2.8	3.5	8.43	6.95	3.8	4.6
IX	13	12	300	25.1	20.3	13.6	14.5	16.25	13.2	8.8	9.4	5.2	4.20	2.8	3.0	6.94	5.6	3.6	4.0
XXI	7	6	180	22.8	16.5	11.8	12.3	13.8	10.0	7.1	7.5	4.7	3.26	2.3	2.4	9.99	7.2	5.2	5.4

3. Changes in Skeletal Calcium.—The percentage of calcium in the bones of the normal rabbits varied from 13.1 to 15.2, and the total calcium per kilo of body weight from 7.6 to 9.5 gm., the average being 8.6 gm. The acid-fed rabbits showed 13.8 to 17.9 per cent of calcium, and 6.2 to 9.1 gm., the average being 7.9 gm., of skeletal calcium per kilo of initial body weight. The last mentioned figures indicate that there was probably some loss of calcium during acid feeding.

4. Changes in Skeletal Carbonate.—The percentage of carbon dioxide in the bones of normal rabbits was 4.8 to 5.2; in the bones of acid-fed rabbits it was 4.7 to 5.6. The total CO_2 per kilo of body weight was 2.8 to 3.1 gm. in the control rabbits; 2.1 to 2.8 in the acid-fed. The ratio of the total carbon dioxide per kilo of initial body weight of the acid-fed rabbits was lower than the lowest normal in six of the eight acid-fed rabbits. From these results it appears that the calcium carbonate of the bones, unlike the phosphate, is reduced by acid feeding. This conclusion is in agreement with that reached by Steenbock, Nelson, and Hart (1914) as the result of metabolism experiments on a calf. The fact that the *percentage* of CO_2 is not reduced in bones of acid-fed rabbits is attributable to the loss of fat which makes the relative proportions of other constituents appear higher.

5. Changes in Bone Fat.—The percentage of fat in the bones of the normal animals was 14.5 to 19.6 averaging, 17.1, and the total fat per kilo of body weight was 8.0 to 11.8 gm. In the acid-fed rabbits, the fat percentage was 3.5 to 14.0 averaging 8.6 or half as high as in controls. The ratio of the total fat per kilo of initial body weight was correspondingly reduced. The reduction occurs even in the early stages of experimental acidosis. Because of this reduction in bone fat, the figures for the percentages of PO_4 and Ca in the bones of acid-fed rabbits are rather higher than normal. The bone powder of the acid-fed rabbits was dryer and visibly less fatty than that of normal rabbits, and it was this appearance which led us to determine the relative fat content.

SUMMARY.

Daily administration of 25 to 75 cc. of 0.25 N hydrochloric acid to rabbits for 1 to 4 weeks had the following effects:

1. Blood plasma bicarbonate was reduced.

2. Phosphoric acid excretion was increased, the increase being in some cases followed by a fall. These observations confirm those of Fitz, Alsberg, and Henderson (1907).

3. Phosphate excretion in the feces was not markedly affected.

4. The muscles lost markedly in phosphorus and potassium and somewhat in sodium.

5. In the skeleton the chief effect was a great reduction in fat content, which fell from an average of 17.1 per cent of the dry weight to 8.6 per cent. Aside from the fat loss, the skeletons of acid-fed rabbits averaged about 10 per cent lighter in dry weight than those of the normals. There was no demonstrable loss of calcium phosphate. There was a definite loss on the average of about one-fifth of the carbon dioxide, indicating that the calcium carbonate is more readily sacrificed in acid intoxication than is the phosphate.

CONCLUSIONS.

The individual variations in muscle and bone are such, that in every constituent determined, the figures for some of the acid-fed rabbits overlap the normal range. Consequently the interpretation of results is not so sharp as could be desired, and it is regrettable that the time available has not permitted the extension of the experiments to a larger number of animals, so that the data could be put on a statistical basis.

The results nevertheless indicate with a fair degree of decisiveness that next to the bicarbonates of the body fluids, the first major reserves of alkali drawn upon in acid intoxication of rabbits are the alkali phosphates, particularly potassium phosphate, of the muscles, and the calcium carbonate of the bones. The bones do not lose calcium phosphate in appreciable amounts.

The above results are only suggestive in their relationship to clinical acidosis. Whether a loss of alkali phosphates from the tissues and calcium carbonate from the bones occurs in the latter remains to be ascertained by accurate determination of mineral balances.

The writer's thanks are due to Dr. Donald D. Van Slyke for his constant advice during the course of this work.

BIBLIOGRAPHY.

Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113.

Folin, O., and Shaffer, P. A., *Am. J. Physiol.*, 1902, vii, 135.

Forbes, E. B., and Keith, M. H., A review of the literature of phosphorus compounds in animal metabolism, *Ohio Agric. Exp. Sta., Technical Bull. 5*, 1914.

Gerhardt, D., and Schlesinger, W., *Arch. exp. Path. u. Pharmakol.*, 1899, xlii, 83.

Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 421.

Givens, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1918, xv, 66.

Goto, K., *J. Exp. Med.*, 1917, xxv, 693.

Mandel, A. R., and Lusk, G., *Deutsch. Arch. klin. Med.*, 1904, lxxxi, 472.

Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.

Rumpf, T., *Berl. klin. Woch.*, 1898, xxxv, 945.

Steenbock, H., Nelson, V. E., and Hart, E. B., *J. Biol. Chem.*, 1914, xix, 399.

Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.

Tenbaum, E., *Z. Biol.*, 1896, xxxiii, 379.

Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxvi, 351.

Walter, F., *Arch. exp. Path. u. Pharmakol.*, 1877, vii, 148.

Weiske-Proskau, H., *Z. Biol.*, 1872, viii, 239.

Wildt, E., *Landw. Versuchssta.*, 1872, xv, 404, cited by Forbes and Keith.

Würtz, A., *Biochem. Z.*, 1912, xlvi, 103.

NOTES ON FOLIN'S DIRECT NESSLERIZATION METHOD FOR THE DETERMINATION OF NITROGEN.

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Two difficulties were encountered in adopting Folin's direct Nesslerization methods[1] for nitrogen determinations in the urine and blood. The first was in making known solutions of pure ammonium sulfate check with the standard when run through as in the procedure for total urinary nitrogen. The second was that after precipitating the blood proteins with *m*-phosphoric acid the filtrate could not be boiled down in a large hard glass test-tube.

In order first to study the problem of the standard solution, two ammonium sulfate solutions were made up, one containing 1 mg. of nitrogen to 1 cc., and the other 1 mg. to 20 cc. All glassware was accurately calibrated, and Ostwald pipettes which delivered between marks were used for the 1 cc. measurements.

Experiment 1.—1 cc. of the more concentrated solution was digested with 1 cc. of the acid mixture in a large hard glass test-tube for 2 minutes after the sulfuric acid fumes had begun to come off, then diluted, and washed into a 200 cc. volumetric flask. 10 per cent sodium hydroxide was added in such amount as to make $\frac{1.1}{5}$ of the volume necessary to neutralize 1 cc. of the acid mixture and 2 cc. for excess alkalinity, and the whole Nesslerized and diluted to volume. 20 cc. of the weaker solution were similarly treated after addition of the acid mixture but omitting the digestion. When compared in the colorimeter the color from the more concentrated solution of ammonium sulfate was more intense than that developed in the weaker solution without digestion. More color was brought out by digestion than could be accounted for by Nesslerization of the ammonium sulfate present so that the weaker solution could not serve as a standard for nitrogen determinations.

[1] Folin, O., and Denis, W., Nitrogen determination by direct Nesslerization, *J. Biol. Chem.*, 1916, xxvi, 437.

Experiment 2.—In order to show whether the error involved in measuring such small amounts of ammonium sulfate or the possible loss in washing into the flask was considerable, 1 cc. of the more concentrated ammonium sulfate solution was put into each of two large hard glass test-tubes and the procedure concluded from this point as before, omitting the digestion. The readings checked exactly.

Experiment 3.—Experiment 2 was next repeated but one sample of the ammonium sulfate was digested for 2 minutes after the white acid fumes had begun to come off, before washing into a 200 cc. volumetric flask. The result was similar to that in Experiment 1. The color developed by the digested ammonium sulfate solution gave a reading of 9.3 when compared with the color from the undigested solution which was used as a standard and set at 10.

Experiment 4.—In order to show that the increase in intensity of the color developed after the digestion of the ammonium sulfate with the acid mixture was due to something in the acid itself, Experiment 3 was repeated using 1 cc. of distilled water in place of the ammonium sulfate solution. No color was developed by the undigested sample while there was a distinct brownish color after Nesslerization of the digested acid mixture.

On account of this impurity of the sulfuric acid now purchasable as C. P. we have found it necessary to make a correction for the color modification due to digestion or to digest our standard solution according to the following procedure.

1 cc. of a solution of purified ammonium sulfate containing 1 mg. of nitrogen to 1 cc. is measured with a calibrated Ostwald pipette into a large hard glass test-tube and digested for 2 minutes with a very low flame, a watch crystal being placed over the top of the tube when sulfuric acid fumes begin to come off. After cooling, distilled water is added, the whole washed into a flask, and set aside until the urine or blood filtrate has been similarly treated. The procedure is then completed as directed by Folin, and the solutions are compared in the colorimeter.

In the determination by direct Nesslerization of the non-protein nitrogen of the blood *m*-phosphoric acid is used as a precipitant. The resulting filtrate is water-clear and does not give the biuret reaction, but on account of bumping we were unable to boil it down in a large hard glass test-tube when the latter was held in the vertical position. If the tube is held just far enough from the horizontal to bring the surface of the liquid half way between the bottom and the mouth of the tube, the microburner flame adjusted so as to be not over $\frac{3}{4}$ inch high with the tip of the

flame 1 cm. from the lowest portion of the tube, and moved away from the center line of the tube toward the edge, as in the accompanying figure, the fluid will boil without loss by bumping or foaming Over 30 minutes will usually be required to boil the 10 cc. of filtrate down to 2 cc. but the tube need not be watched more than occasionally. When blackening begins the tube is turned upright and the remainder of the procedure carried out as in the case of urine. At this point, however, the precaution should be

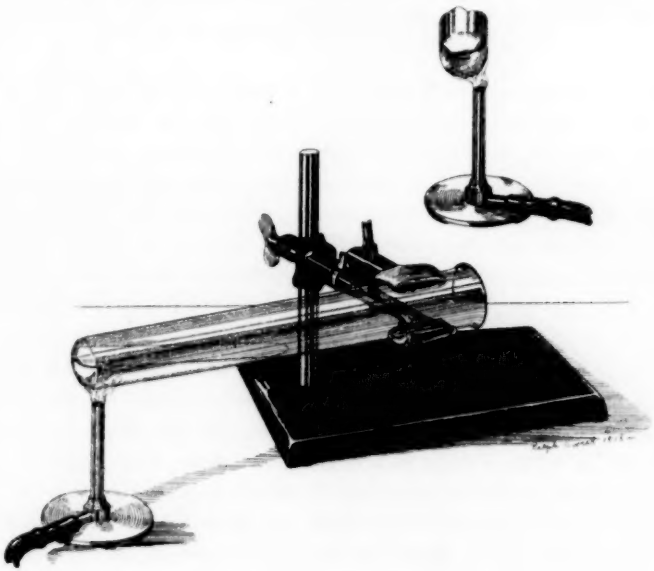

FIG. 1.

taken of placing the tip of the flame well over to one side, otherwise bumping and loss will occur. While keeping it in contact with the tube the flame should at the same time be lowered as far as is consistent with the continued digestion of the blackening filtrate, otherwise there will be too great a loss of sulfuric acid fumes and a consequent solidification of the material in the tube as digestion is completed. With the greatest care at the end of digestion solidification will sometimes occur and ruin the determination by making it difficult to wash the material quantitatively into a flask.

This can be prevented by raising the watch crystal slightly on one side and cautiously introducing distilled water with a capillary pipette and rubber nipple drop by drop down the side of the tube as soon as blackening disappears and the solution clears. Sputtering but no loss of material will occur. The remainder of the procedure is carried out as directed by Folin.

With these slight modifications we have found the method for determination of total urinary nitrogen and non-protein nitrogen of the blood by direct Nesslerization to be accurate and to effect a great saving of time and chemicals.

ON THE DETERMINATION OF UROBILIN IN URINE.

PRELIMINARY REPORT.

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To the long list of methods of clinical investigation from which modern medical science has benefited may be added the determination of an increased quantity of urobilin in the urine of patients suffering from liver complaints.

Practically all modern investigators employ Schlesinger's method, which is based on the fact that even the smallest quantity of urobilin on the addition of certain zinc salts in an alcoholic solution produces a green fluorescence. As a matter of fact Jaffé had already called attention to this.

Schlesinger employed equal portions of urine and a 10 per cent suspension of zinc acetate in absolute alcohol. Hildebrandt points out that the reagent must be well shaken immediately before using, as a saturated solution of zinc acetate in absolute alcohol only contains 2 per cent and that to obtain the reaction it is sometimes necessary to work with large quantities of zinc acetate. Furthermore, he allows the mixture of urine and the reagent to stand for 12 to 24 hours before filtering in order that the urobilinogen present may be oxidized to urobilin. The same result is obtained instantly by the addition of minimum quantities of iodine.

In using Schlesinger's test in this way it was strikingly clear to us that the reagent, in the course of a few days, changed its appearance. While the *freshly* prepared suspension produced by finely pulverized zinc acetate and absolute alcohol was easily mixed by shaking, and on measuring gave practically uniform results with regard to the contents of zinc acetate in 10 cc., *this was not the case when the reagent was allowed to stand for a few days.* A freshly prepared suspension from which, after carefully

shaking, we measured 10 cc. into a porcelain bowl, evaporated the alcohol on a steam bath, and dried the remainder to a constant weight at 70°, gave as an average result of three tests 0.857 gm. of anhydrous zinc acetate, which corresponds to about 1.025 gm. of the hydrous zinc acetate used $(CH_3COO)_2Zn2H_2O$. A series of tests carried out in the same way after a lapse of 8 days gave, on the other hand, highly variable values from 0.27 to 0.82 gm. of zinc acetate.

On closer consideration the reason for this was obvious. The powdered zinc acetate had in the course of the 8 days changed into a coarse, crystalline, monoclinic zinc acetate which it was very difficult to shake up and which was very quickly precipitated.

To avoid the difficulty in the case of the prepared "Schlesinger's reagent" we decided to work, in all our experiments, with a weighed quantity of zinc acetate and a measured quantity of alcohol and urine.

Our method of procedure was as follows: Into one test-tube we weighed 1 gm. of zinc acetate and added to it 10 cc. of absolute alcohol; in another test-tube we measured 10 cc. of urine to which we added three drops of a 5 per cent alcoholic solution of iodine. We then carefully mixed the contents of the two test-tubes by repeated decantations until all the zinc acetate was dissolved. We finally filtered the precipitate and examined the filtrate for fluorescence in daylight falling from behind the observer through a test-tube 16 mm. wide.

On examining the urine of a number of patients suffering from different liver complaints, we obtained in this way very fine fluorescence results, but later on examining the urine of a number of normal persons and also of patients suffering from different surgical complaints, without any symptoms of liver involvement and with normal temperature, we found in the great majority of cases a very distinct fluorescence. It is, of course, well known that the urine of normal, healthy persons contains small quantities of urobilin. Gerhardt found 0.0074 to 0.0079 gm., Friederich Müller up to 20 mg., Saillet, 30 to 130 mg., and G. Hoppe-Seyler 80 to 140 mg. in the 24 hour urine. In other words the method proved too delicate in working with the exact quantities of reagent and urine.

Gregersen has, it is true, examined 800 patients apparently free from liver complaints and 50 normal persons, altogether over 5,000 urobilin tests, with Schlesinger's reagent and never found one positive reaction. A possible explanation of this striking disparity may lie in the fact that he, like all earlier investigators, used Schlesinger's reagent without considering the small quantity of zinc acetate he *might* in this way happen to work with.

In order to test the importance which the quantity of zinc acetate plays in the reaction we carried out the following experiment.

10 cc. of a slightly acid urine containing urobilin were measured into each of nine test-tubes. To each were added three drops of a solution of iodine. 10 cc. portions of absolute alcohol were measured into nine other test-tubes to which were added quantities of zinc acetate varying by 0.1 gm. from 0.9 down to 0.1 gm.

After careful mixing and filtering the experiments showed that the fluorescence was apparently unchanged in strength from 1 to 0.50 gm. Below that it decreased steadily until at 0.20 to 0.10 gm. it had to be considered as 0. Different urines, however, proved somewhat variable in this respect, but in the case of all of them it may be said *that the fluorescence was unaffected by varying the amount of zinc acetate from 0.5 gm. upwards, but that decreasing the quantity below 0.5 gm. diminishes the fluorescence obtained.*

We then examined the effect of alcohol concentration on the reaction.

As will be seen from Table I *the fluorescence decreases with the decreasing alcohol percentage.*

To complete the experiment the quantity of iodine was varied from half to threefold. This produced no visible change.

There seems to be some diversity of opinion with regard to the importance of the reaction of the urine. Hildebrandt states that highly concentrated acid urines do not give a reaction even when rich in urobilin, and, like most other investigators, he considers that a very weak alkaline reaction produces the best fluorescence results, while, for instance, Gregersen states that the reaction of the urine is of no importance whatsoever.

We decided, therefore, to try to throw some light on this point also by making the reaction on urobilin-containing urines to which were added N hydrochloric acid, N sulfuric acid, N acetic acid, or N solution of ammonia, in varying quantities but in such a way that the total volume remained constant.

TABLE I.

No.	Urine.	Iodine.	Zinc.	Water.	Alcohol.		Fluorescence.
					Absolute.		
	cc.	*drops*	*gm.*	*cc.*	*cc.*	*per cent*	
I	1	1/3	0.20	1	8	80	Distinct.
II	1	1/3	0.20	2	7	70	"
III	1	1/3	0.20	3	6	60	"
IV	1	1/3	0.20	4	5	50	"
V	1	1/3	0.20	5	4	40	Decreasing.
VI	1	1/3	0.20	6	3	30	"
VII	1	1/3	0.20	7	2	20	Very slight.
VIII	1	1/3	0.20	8	1	10	0
IX	1	1/3	0.20	9	0	0	0

TABLE II.

No.	Urine.	Solution of iodine.	N HCl	Water.	Zinc acetate.	Absolute alcohol.	Filtrate.	
							Acidity, about	Fluorescence.
	cc.	*drops*	*cc.*	*cc.*	*gm.*	*cc.*		
I	5	2	0	5	0.50	10	Very slightly acid.	Strong.
II	5	2	1	4	0.50	10	0.05 N	"
III	5	2	2	3	0.50	10	0.1 N	Distinct.
IV	5	2	3	2	0.50	10	3 0.05 N	Slight.
V	5	2	4	1	0.50	10	0.2 N	Very slight.
VI	5	2	5	0	0.50	10	0.25 N	0

Table II shows the effects of the acids employed, Tables III and IV those of the ammonia solution.

The tests were made on slightly acid urobilin-containing urines.

The fluorescence of the filtrate decreases with the increasing quantity of the hydrochloric acid and disappears at the point where the zinc acetate is converted into zinc chloride, 0.50 gm. of zinc acetate requiring about 4.6 cc. of N HCl.

$$(CH_3COO)_2Zn - 2H_2O + 2HCl = ZnCl_2 + 2CH_3COOH + 2H_2O.$$

In other words, in acid-reacting urines zinc chloride does not give any reaction, whereas a mixture of zinc acetate and zinc chloride does. This can be proved by the non-fluorescing filtrate (Test VI) fluorescing on the addition again of 0.50 gm. of zinc acetate and then filtering.

Sulfuric acid gave quite the same result, *whereas acetic acid added in equivalent quantities had no visible influence on the strength of the fluorescence.*

It was extremely interesting to observe the influence of ammonia on the reaction.

The urine employed had a slightly acid reaction and gave the urobilin reaction in a dilution of 1:20. The point where the filtrate shows the weakest fluorescence probably coincides with

TABLE III.

No.	Urine.	Solution of iodine.	N NH_3	Water.	Zinc acetate.	Absolute alcohol.	Fluorescence.
	cc.	*drops*	*cc.*	*cc.*	*gm.*	*cc.*	
I	5	2	0	5	0.5	10	Strong.
II	5	2	1	4	0.5	10	Slight.
III	5	2	2	3	0.5	10	Very slight.
IV	5	2	3	2	0.5	10	" "
V	5	2	4	1	0.5	10	Slight.
VI	5	2	5	0	0.5	10	Strong.

the point where the solution holds the least quantity of zinc salt dissolved. On repeating the experiment with other urines the same conditions were discovered: *an increasing amount of ammonia gave first a decreasing and then again an increasing fluorescence*, but the minimum fluorescence of the different urines required the addition of a varying quantity of ammonia; in the same way the strengthening of the fluorescence required varying quantities of ammonia. In order to obtain this distinctly it was necessary in the case of urines rich in urobilin to use smaller quantities of urine, for instance 2 cc. instead of 5; and, in order to obtain the same total volume, an increased addition of water. Table IV shows a series of such experiments.

In other words ammoniacal urines ought always to be acidified with acetic acid before making the test as it is impossible to know in advance whether the quantity of ammonia is such as to prevent

entirely the reaction or to lead to an erroneous estimate of the quantity of urobilin. Gregersen's statement that the test can be made equally well on urines which have stood for several days may be wrong if the urine contains urea-decomposing bacteria. In the case of highly acid urines it is advisable to make alkaline with ammonia, then to acidify with acetic acid.

In order to discover whether the method could be employed to distinguish between normally and pathologically increased quantities of urobilin, we carried out a series of dilution tests. The aim of these was to ascertain how much it is necessary to dilute normal urine to avoid getting a positive reaction, and to find whether in liver complaints the quantity of urobilin is of such

TABLE IV.

No.	Urine.	Iodine solution.	N NH_3	Water.	Zinc acetate.	Absolute alcohol.	Fluorescence.
	cc.	*drops*	*cc.*	*cc.*	*gm.*	*cc.*	
I	2	1	1	7	0.5	10	Strong.
II	2	1	2	6	0.5	10	"
III	2	1	3	5	0.5	10	"
IV	2	1	4	4	0.5	10	Slight.
V	2	1	5	3	0.5	10	0
VI	2	1	6	2	0.5	10	Slight.
VII	2	1	7	1	0.5	10	Strong.
VIII	2	1	8	0	0.5	10	"

magnitude that the urine allows of greater dilution before the reaction fails to appear.

From our investigations into the importance of the alcohol percentage on the reaction we knew that in these tests we had to be careful that the alcohol percentage did not fall below about 40. We had likewise to make sure that the amount of zinc acetate was not too small. Fischler, who attempted such dilution experiments to form an estimate of the quantity of urobilin, made the fatal mistake of diluting the fluorescing filtrate with water, which prevents fluorescence even with very slight dilution. It appears, however, from the following experiment that by again increasing the alcohol percentage to the original quantity a distinct fluorescence reappears which is proportional in intensity to the amount of urine present. 10 cc. of a strongly fluorescing

filtrate are diluted with 10 to 15 cc. of water. The fluorescence disappears; then on the addition of 10 to 15 cc. of absolute alcohol a distinct fluorescence reappears. We cannot confirm Gregersen's observation that the fluorescence suddenly disappears at a certain point of dilution, if care is taken to keep the alcohol concentration at about 40 to 50 per cent.

In carrying out the dilution experiments in the manner stated below we found a quite uniformly decreasing fluorescence which in the case of urines rich in urobilin could still be observed with a dilution of 1:100. The difficulty at this point is to decide when the fluorescence may be described as non-observable, as even with a greater dilution we could determine a quite slight fluorescence, either by looking at the solution in a test-tube held horizontally, or by throwing a ray of light transversely across the glass with a convex lens.

In our experiment, however, we never availed ourselves of these accessories, and when we describe the fluorescence as 0, it means that the fluorescence was not observable in daylight falling from behind the observer through a test-tube 16 mm. in diameter.

We carried out these dilution tests in the following manner. 10 cc. of urine were measured drop by drop into test-tube A and to this were added three drops of a 5 per cent alcoholic solution of iodine. After shaking up, 1 cc. of this is measured into test-tube B.

I. To test-tube A, which now contains 9 cc. of urine, are added 9 cc. of absolute alcohol and 1 gm. of zinc acetate, and after complete solution of the zinc acetate the contents are filtered through a plain filter (9 cm. in diameter) into a test-tube of the width mentioned above (9 cc. of urine to 18 cc. of total volume; dilution $\frac{1}{2}$).

II. If the filtrate fluoresces, to test-tube B are added 3 cc. of water, 1 cc. of a clear 20 per cent solution of zinc acetate in water, and 5 cc. of absolute alcohol. After mixing, it is filtered (1 cc. of urine in 10 cc. of total volume; dilution $\frac{1}{10}$).

III. If the filtrate fluoresces, 5 cc. of urine are measured out drop by drop; to this are added 5 cc. of water and two drops of solution of iodine. From the mixture 1 cc. is measured into another test-tube and mixed with 3 cc. of water, 1 cc. of 20 per

cent zinc acetate solution, and 5 cc. of absolute alcohol, and then filtered. (0.5 cc. of urine in 10 cc. of total volume; dilution $\frac{1}{20}$.)

IV. If the filtrate fluoresces, 5 cc. of the urine are measured drop by drop into 15 cc. of water and two drops of iodine. From the mixture 1 cc. is measured, and the procedure is the same as in III (0.25 cc. of urine in 10 cc. of total volume; dilution $\frac{1}{40}$).

In working in this way one is always certain of (1) *having a sufficient excess of zinc acetate*, (2) *sufficiently large and constant alcohol concentration*. Under these conditions it is possible to estimate the amount of urobilin in a urine. In a number of patients suffering from liver complaints the fluorescence could even be determined in dilutions of $\frac{1}{40}$ to $\frac{1}{80}$, and in certain cases even with still greater dilutions. In a very few out of the 50 normal patients whose urine we have examined we have on one or two occasions seen the fluorescence in a dilution of $\frac{1}{10}$, and we should therefore be inclined to consider a fluorescence in a dilution of $\frac{1}{20}$ as the lowest limit for a pathologically increased urobilinuria. But to decide this point more definitely a far greater number of investigations is necessary than we are able to carry out at present.

Perhaps it would not be out of place here to mention that the evaporation experiments conducted by Gregersen are based on a misunderstanding, as the urobilin is obviously a thermolabile substance.

We have made the following experiments. 250 cc. of slightly acid urobilin-containing urine (the fluorescence apparent in a dilution $\frac{1}{40}$) were heated in a glass vessel in a water bath. The temperature of the urine reached its maximum of 95° in the course of 20 minutes. The urine was kept at this temperature for 1 hour. After restoring the water to its original weight from the loss it had undergone from evaporation and cooling, we conducted the urobilin test in the usual way. The fluorescence was now only visible in a dilution $\frac{1}{10}$. After remaining another hour on the water bath, restoring the water, and cooling as before, the fluorescence could only be seen in a dilution $\frac{1}{2}$ and after a further hour's heating the fluorescence could not be observed at all.

CONCLUSIONS.

The methods formerly employed for the quantitative determination of urobilin have either, as in the case of isolation and weighing, proved far too complicated for clinical use or, as in the case of spectroscopic methods of examination, not sufficiently delicate. By the dilution tests, here described, one, of course, does not obtain a definite figure for the quantity of urobilin present, but it is possible by a test, taking only a few minutes, to estimate sufficiently near for clinical use, how large quantities of urobilin a urine contains. We hope that further experiments with our method will be able to throw fresh light on several points related to it; for instance, on the importance of the concentration of the urine, the contents of pathological components, the variations in quantity of the substances usually present, the importance of the food, consumption of alcohol, and several other things which we hope later to be able to help in clearing up.

STUDIES ON THE NUTRITION OF FISH.

EXPERIMENTS ON BROOK TROUT.*

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Metabolic studies on aquatic animals have not been numerous. The inherent difficulties of collecting the excreta and measuring accurately the consumed food, both of which are soluble in the water, have doubtless made this subject unattractive to investigators.

In undertaking a nutritional study on the brook trout—a fish remarkably sensitive to slight changes in its environment—the first problem to settle was the practicability of keeping these animals in small aquaria. The trout thrive in rapid streams, and the problem would evidently be beyond solution if they could not be kept in good health in a limited quantity of water. Experience has shown that with sufficient aeration, trout can be kept in as little as 4 or 5 liters of water, which need not be changed but once every 48 hours, or even at longer intervals. Under such circumstances the trout will not only remain in good condition, but as the experiments here recorded show, will gain weight.

Large museum jars, about 12 inches in diameter, were used as aquaria, and these offered the fish considerable room for swimming, and could be kept very clean. The jar was closed by

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a cover which could be clamped tightly to the bottom, and, by means of a flat rubber ring, the joint made air-tight. Two round holes drilled in the cover of the jar were fitted with rubber stoppers. Through one of these, the aeration tube was passed, while in the other a specially designed cup was inserted containing a measured quantity of standard acid. Compressed air was blown through the water, and was dissipated as a fine spray by the aeration tube. The air escaping from the jar bubbled through the standard acid contained in the cup. This precaution was taken in order to avoid any possible loss of ammonia from the water by the vigorous current of air.

The contents of the cup were emptied every 24 hours and titrated with 0.01 N sodium hydroxide. As no changes in the quantity of acid have been found in the course of many trials, this practice was discontinued as unessential to the accuracy of the experimentation.

The aquaria were submerged in a trough of galvanized iron through which a constant circulation of water of fairly uniform temperature was maintained. The temperature of the aquaria in which the trout sojourned was thus regulated. The water used in these experiments was exceptionally pure, coming directly from the excellent filters installed in the New York City Aquarium; no sediment was formed by this water even upon standing several weeks.

The trout which were experimented on were weighed at the beginning and close of each period. Different methods have been tested for obtaining the accurate weight of the fish, and finally the following method was adopted: The trout was picked up with a small fine net, the adhering water shaken off, and the trout allowed to drain for about half a minute. The fish was then transferred cautiously to an aluminum can partly filled with water, the weight of which was accurately determined. With practice it was possible to perform this operation without splashing a drop of water. The can was closed and again weighed with the trout, whose weight was thus gotten by difference. Though the method is not free from certain defects, it had two important advantages over every other method tried, in that, in the first place, no injury was done to the trout, and second, with the animal securely in the can, the weighing could be done lei-

surely Of course it is assumed that the amount of moisture adhering to the animal has been the same at each weighing. Though this assumption is arbitrary, it has been found by weighing the animals several times in succession, that the extreme differences do not vary more than 0.5 per cent. The balance employed in all weighings was sensitive to a mg. with a load of 1.5 kilos.

Feeding the trout was unquestionably the most difficult, and yet the most essential step in the process of accurately measuring their metabolism. The effort, therefore, was made to teach the fish to take their food directly from forceps. Wherever this was feasible, the rest of the experimental procedure was quite simple. In a few instances I was actually successful in so training the trout that they would come to the edge of the aquaria and leaping out of the water, snap the food held with pincers. In this way the washing out of soluble constituents of the food by the water was entirely prevented.

The food was kept in small weighing bottles, and the amount consumed was determined by the difference in the weight before and after feeding. The method of feeding the trout *ad libitum* had the advantage also that at no time were there unconsumed particles of food left in the aquaria which might favor contamination of the water, and thus greatly affect the significance of the results.

Unfortunately this method of direct feeding could not always be utilized, as will be shown in a later section. The food was generally prepared in large quantity and stored in a frozen condition. The contents of every jar were carefully analyzed, and their composition was checked at least twice in the course of an experiment. Portions of this stock food—enough for several feedings—were put in weighing bottles and kept in the ice chest in the laboratory.

Usually 48 hours after feeding, the trout was removed to a jar with a fresh supply of water, and the old water containing the solid and dissolved excreta of the preceding period was filtered and prepared for analytical treatment.

Large aluminum tumblers, the bottoms of which were perforated with a number of fine holes, were used for this purpose, the tumblers serving as Gooch crucibles. The tumblers were provided with a thick pad of fine glass wool, dried in the oven, and

weighed. The contents of the aquaria were siphoned into the tumbler and filtered through the glass wool, with the aid of suction, into a large bottle. The glass wool was found very efficient in retaining even minute particles floating in the water, but the filtered water showed invariably a distinct turbidity. The tumbler with the solid excreta retained in the glass wool was again dried and weighed; thus the weight of dry feces was gotten by difference.

It may be mentioned that the sides and bottom of the aquaria were thoroughly cleaned with a rubber-tipped rod, and the wash water added to the filtrate. This was acidified with a few drops of sulfuric acid and evaporated to a small bulk. As a rule, the final volume was made up exactly to 1 liter.

In view of the large quantities of water that it was necessary to handle, the matter of evaporation presented certain technical difficulties. Originally it was attempted to carry out the evaporation at a low temperature (50°C.) with the aid of a rapid current of hot air. This method was very cumbersome, requiring much time. Blank experiments have shown that there was no particular advantage derived from the use of a low temperature. A large steam bath was therefore installed, accommodating a number of evaporating dishes (white enamel) of about 12 liters capacity each. The steam bath was set in the apparatus previously used for evaporating by means of the current of dry air, so that the two methods could be combined at will. Very large quantities of water could in this way be quickly condensed to a small bulk. The concentrated water was transferred to a volumetric flask, the evaporating dish thoroughly rinsed with fresh water, and the quantity brought up to a definite volume. This concentrated water was again filtered through asbestos to remove such particles as may have gone through the glass wool. The quantity was negligible, but the amount accumulated in the course of an experiment was analyzed and added to the feces. Aliquot portions of the perfectly clear water were used for analysis.

In choosing fine glass wool as a means of separating the solid excreta from the water, two important considerations were borne in mind; owing to the small quantity of feces available, it was very difficult to analyze it, and particularly to obtain a uniform sample. It was urgent, therefore, first to mix the feces

with some other material to increase its bulk without interfering with the analysis, and secondly, to make the grinding of the feces possible. The use of glass wool for this purpose suggested itself after a number of different things had been tried with little or no success. The glass wool proved particularly ideal as it served as a filtering medium, and could be ground to a thin powder, helping to reduce the feces to a state of extremely fine subdivision and uniform distribution.

These powders in which the glass represented many times the bulk of the feces, were kept in weighing bottles and were easily sampled for analysis. Nitrogen and fat determinations were made on weighed portions in the usual manner. It may also be mentioned that blank experiments were performed, and the analytical data furnished corrections for the dissolved excreta and feces which were found in experiments with trout.

Experiments in Fasting.

As a preliminary to the feeding experiments, a number were performed on fasting trout (Table I). It has been found that the alimentary tract frees itself of all excreta from previous feeding in 48 hours. Minute quantities were sometimes eliminated also during the next 24 hours, but this was invariably negligible; the fasting, therefore, was generally started 48 hours after the last meal. The first protracted fast was performed with a trout weighing 102.9 gm. (F-1). In the course of the 4 weeks of the experiment, no solid excreta were eliminated. The water in the aquarium remained remarkably clean for days, so that it could be changed at long intervals. The nitrogen eliminated in the water was determined for 7 day periods. After 28 days of fasting, the trout weighed 95.7 gm. or 7.2 gm. less than at the start. The nitrogen eliminated during the first week of fasting was 67.2 mg., but the quantity diminished from week to week, only 45.1 mg. being eliminated during the last week. The average elimination of nitrogen per day and per kilo of weight was 81 mg. In the course of 4 weeks 233 mg. of nitrogen were lost. It is evident, therefore, that about one-fifth of the body loss was at the expense of the protein.

The next two experiments were made with trout which had been kept previously in the stock tank and fed freely. Whe

TABLE I.

Experiments in Fasting.

Experiment No.	Period of fasting.*	Average temperature of water.	Weight.		Loss in weight.	Total N excreted.	N excretion per day.	N excretion per day and per kg.	Fat excretion (total).	Fat excretion per day and per kg.
			Initial.	Final.						
		°C.	gm.	gm.	per cent	gm.	gm.	gm.	gm.	gm.
F-1	I.25–II.1	16.3	102.9	—	—	0.0672	0.0096	0.0941	—	—
	II.1–II.8					0.0629	0.0090	0.0900	—	—
	II.8–II.16		—	—	—	0.0583	0.0073	0.0745	—	—
	II.16–II.23			95.7	7.0	0.0451	0.0065	0.0670	—	—
						0.2335	0.0081	0.0813		
F-2	VI.4–VI.11	17.2	52.2	49.7	4.6	0.0464	0.0072	0.1419		
	VI.11–VI.18		49.7	49.1	1.3	0.0243	0.0035	0.0710		
						0.0042 (Feces)			0.0043	0.0061
					5.9	0.0749	0.0054	0.1055		
F-3	VI.5–VI.12	17.2	82.4	81.0	1.7	0.0695	0.0099	0.1212		
						0.0063 (Feces)			0.0082	0.0143
						0.0758	0.0108	0.1322		
F-4	II.10–II.17	16.7	91.1			0.0965	0.0138	0.1559		
	II.17–II.24			82.6	9.3	0.0458	0.0065	0.0793		
						0.1423	0.0102	0.1171		
F-5	IV.22–IV.30	16.6	57.4	56.3	1.9	0.0415	0.0052	0.0916		

*The Roman numerals are used in the tables to designate the month of the year, the date being indicated by the arabic numerals.

transferred to the experimental aquaria, they vomited much undigested food, and for a few days continued to eliminate large amounts of feces. Trout F-2 in the 1st week of fasting lost 4.6 per cent of its weight, eliminating 141 mg. of nitrogen per day and kilo. This very high nitrogen elimination was followed by a decided drop in the next week, when it was only 71 mg., the loss in weight at the same time having been reduced to 1.3 per cent. During the entire 2 week period of fasting 5.9 per cent of the body weight was lost, and the daily nitrogen elimination per kilo of fish was 105 mg. The very high nitrogen elimination during the first few days of fasting which, in this case was preceded by abundant and unrestricted feeding, will be observed in several other experiments. This condition is met with also in the case of the higher vertebrates.

In Experiment F-3 it will likewise be observed that the nitrogen elimination reached a very high level of 132 mg. per day and kilo, but the fast was not continued further with this animal.

Experiment F-4 presents essentially the same picture. This trout was used in a long feeding experiment, and for weeks it was fed regularly and *ad libitum*. It was then subjected to a fast of 2 weeks duration, during which time it had lost 9.3 per cent of its weight. The nitrogen eliminated in the 1st and 2nd week shows that it was twice as large during the former, being 155 mg. of nitrogen per day and per kilo for the first 7 days, and only 79 mg. for the next 7 days. This large loss of nitrogen observed invariably upon changing from an abundant diet to fasting, especially in the case of Trout F-4 which for a month previous to the fast had been eliminating a fairly constant amount of nitrogen daily, brings up again the question of the circulating protein which, when the income of new food material is stopped, is the first to be consumed.

Experiment F-5 like the first of the series is interesting because this trout was not on an abundant diet previous to the fast. Its nitrogen elimination for 8 days was 91 mg. per day and kilo of body weight. We may regard, therefore, the elimination of 80 to 90 mg. per day and per kilo as the normal nitrogen catabolism of the brook trout.

Feeding Raw Beef Heart.

Raw beef heart cut in fine strips about 1 cm. long was fed directly from forceps. The results of an experiment which lasted over 2 months are recorded in Table II. During this time the excreta for every 48 hours were collected and analyzed separately. In the course of the 1st month, it will be seen, the trout gained 77.3 per cent in weight. It consumed 85.34 gm. of beef heart, and the excretion from day to day showed remarkable constancy. It will be noted also that the amount of nitrogen in the feces was almost invariably 10 per cent of the dry fecal matter, and this proportion coincides with that found in the feces of mammals on a pure meat diet. The dry feces constituted about 5 per cent (cf. Table VI) of the dry material ingested with the food. The utilization of the food—protein and fat—has been almost constant throughout this part of the experiment. Of the 2.8981 gm. of nitrogen fed, 0.9329 gm. was retained (cf. Table II, total N intake and total N eliminated). In the absence of experiments on the respiratory exchange, the retention of fat could not, of course, be worked out. Considering the amount lost with the feces, 96.1 per cent of the consumed protein was utilized, and 94.5 per cent of the fat. Not only has the utilization of the food through digestion been good, but as the "index of growth" (ratio between increase in weight and quantity of consumed food per kilo and per 24 hours) shows, it furnished a considerable proportion of its material to the building up of the body tissues.

The consumption of the trout per day and kilo of weight amounted to 37.5 gm. of food, while the increase in body weight per day and kilo was 17.4 gm. In other words, 46.5 per cent of the food material was added to the organism (see Table VI). The trout was then allowed to fast for a fortnight (F-4, Table I), in order to find out how this would affect the digestive power and general condition of the animal whose metabolic exchange has been carefully established by the previous study.

Upon resuming feeding, the trout gained 15.7 per cent in 2 weeks. During the first 4 days of realimentation, the small amount of nitrogen in the water (urine) is marked. During the first part of the experiment, the urinary nitrogen represented on the average 63.9 per cent of the total nitrogen consumption,

TABLE II.

Experiment 000.5. Raw Beef Heart.

Average temperature	Period.	Initial or final weight.	Change in weight.	Amount of food.	N intake.	Fat intake.	Dry feces.	N in feces.	Fat in feces.	N in water (urine).	Total N eliminated.	N in feces in relation to N intake.	Fat in feces in relation to fat intake.	N in water in relation to N intake.
°C.		gm.	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
16	I.9–I.13	51.4	—	6.07	0.2057	0.1718	0.0625	0.0153	0.0328	0.1080	0.3263	2.84	7.30	52.5
17.3	I.13–I.17	—	—	9.80	0.3324	0.2778	0.0840			0.2030				61.1
17.9	I.17–I.21	—	—	9.10	0.3079	0.2572	0.0982	0.0269	0.0302	0.2085	0.4774	3.81	5.12	67.7
16.3	I.21–I.25	—	—	11.74	0.3983	0.3328	0.1568			0.2420				60.8
15.8	I.25–I.27	—	—	5.57	0.1890	0.1578	0.0792	0.0068	0.0099	0.1211	0.1279	3.60	6.21	64.1
16.8	I.27–I.29	—	—	5.73	0.1995	0.1624	0.0862	0.0085	0.0094	0.1314	0.1399	4.27	5.79	65.9
15.1	I.29–I.31	—	—	5.94	0.2015	0.1683	0.0829	0.0084	0.0097	0.1338	0.1422	4.17	5.75	66.4
16.2	I.31–II.2	—	—	5.98	0.2029	0.1693	0.0835	0.0085	0.0102	0.1338	0.1423	4.19	6.02	65.0
15.3	II.2–II.4	—	—	5.48	0.1859	0.1552	0.0692	0.0075	0.0082	0.1208	0.1283	4.03	5.22	65.9
16.4	II.4–II.6	—	—	7.43	0.2521	0.2105	0.1060	0.0105	0.0093	0.1586	0.1691	4.17	4.42	62.9
16.0	II 6–II.8	—	—	7.13	0.2409	0.2021	0.0985	0.0104	0.0061	0.1658	0.1762	4.32	3.01	68.8
15.7	II.8–II.10	91.1	—	5.37	0.1820	0.1521	0.1050	0.0106	0.0139	0.1250	0.1356	5.82	9.13	68.7
16.4	I.9–II.10	39.7	+77.3	85.34	2.8981	2.4173	1.1120	0.1134	0.1397	1.8518	1.9652	3.91	5.78	63.9
16.7	II.10–II.24	82.6	−9.33	Fasting.										
17.5	II.24–II.26	82.6	—	4.48	0.1613	0.1219	0.0430	0.0048	0.0039	0.0641	0.0689	2.98	3.20	39.7
13.8	II.26–II.28	—	—	5.04	0.1814	0.1372	0.0680	0.0076	0.0061	0.1038	0.1114	4.19	4.45	57.2
15.0	II.28–III.2	—	—	4.06	0.1462	0.1105	0.0905	0.0082	0.0117	0.1551	0.1633	5.61	10.59	106.1
14.6	III.2–III.4	—	—	6.03	0.2038	0.1293	0.1210	0.0121	0.0169	0.1705	0.1826	5.94	13.07	83.2
15.4	III.4–III.6	—	—	4.40	0.1584	0.1198	0.1090	0.0107	0.0127	0.1318	0.1425	6.76	10.60	83.2
16.6	III.6–III.8	—	—	5.09	0.1832	0.1385	0.1160	0.0113	0.0109	0.1292	0.1405	6.17	7.89	70.5
15.2	III.8–III.11	95.5	—	6.54	0.2354	0.1780	0.1390	0.0113	0.0151	0.1732	0.1845	4.80	8.48	73.6
15.4	II.24–III.11	12.9	+15.7	35.64	1.2695	0.9352	0.6865	0.0660	0.0773	0.9277	0.9937	5.20	8.27	73.08

but during the first and second 48 hours following the fast, it was 39.7 and 57.2 per cent respectively. There was thus undoubtedly a retention of nitrogen. As the elimination for the next 48 hours shows, this retention was only temporary.

During this third 2 day period, the urinary nitrogen represented 106.1 per cent of the consumed nitrogen. In other words the nitrogen ingested since the fast was broken, was not excreted until 6 days later. The nitrogen elimination then gradually diminished, but even after 2 weeks, it was still 10 per cent higher than in the first part of this experiment.

An examination of the feces also revealed that the amount of dry excreta has become 50 per cent greater than during the preceding period, and furthermore that the feces became very fatty; in fact, from the 5th until the 10th day of resumed feeding, the per cent of fat in the feces was extremely high; this apparently was due to delayed elimination of undigested fat rather than to a progressive impoverishment of the digestive functions. In this respect there is almost a direct relationship to the excretion of urinary nitrogen. The utilization of nitrogen during these 2 weeks was 94.8 per cent, not essentially different from the degree of utilization in the prefasting period, but the fat utilization was only 91.7 per cent.

That the general condition of the trout was impaired by the fasting, is indicated best by the lowering of the "growth index" (see Table VI). During these 15 days the trout received 26.7 gm. of raw beef heart per day and per kilo of body weight, but it gained only 9.73 gm. in weight; in other words, the "growth index" was 36.5 per cent as compared to 46.5 per cent in the earlier part of the experiment.

The experiment recorded in Table III presents much similarity to the preceding one.

Six small fingerlings, weighing all together only 33.6 gm., were used. During the first part of the experiment, lasting 26 days, these small trout were fed thirteen times and their excreta were collected and separately analyzed four times during that period. A glance at Table III will show that the animals consumed 25.41 gm. of the raw beef heart, and gained 11.13 gm. in weight. The quantity of dry feces eliminated in that time was 3.52 per cent of the total dry ingested food, and contained 21 mg. of nitrogen.

TABLE III.

Experiment 000.6. Raw Beef Heart.

Average temperature.	Period.	Initial or final weight.	Change of weight.	Amount of food.	N intake.	Fat intake.	Dry feces.	N in feces.	Fat in feces.	N in water (urine).	Total N eliminated.	N in feces in relation to N intake.	Fat in feces in relation to fat intake.	N in water in relation to N intake.
°C.		gm.	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
17.1	I.19–I.27	33.6	—	4.74	0.1609	0.1344	0.0368	0.0036	0.0056	0.1344	0.1380	2.24	4.17	83.5
15.9	I.27–II.2	—	—	5.09	0.1727	0.1443	0.0540	0.0047	0.0056	0.1245	0.1292	2.72	3.88	72.1
16.2	II.2–II.8	—	—	7.23	0.2455	0.2051	0.0566	0.0050	0.0069	0.1730	0.1780	2.04	3.36	70.5
16.1	II.8–II.14	44.7	—	8.35	0.2871	0.2404	0.0864	0.0079	0.0092	0.1962	0.2041	2.75	3.41	68.4
16.3	I.19–II.14	11.1	+33.4	25.41	0.8662	0.7239	0.2338	0.0212	0.0273	0.6281	0.6493	2.45	3.77	72.5
	II.14–II.18			Fasting.										
16.3	II.18–II.23	34.8	—	2.84	0.1022	0.0782	0.0375	0.0037	0.0067	0.1074	0.1111	3.62	8.57	105.1
17.5	II.23–II.27	—	—	4.00	0.1440	0.1104	0.0763	0.0074	0.0099	0.0982	0.1056	5.14	8.97	70.0
14.8	II.27–III.3	—	—	4.72	0.1699	0.1305	0.0650	0.0066	0.0097	0.1082	0.1148	3.89	7.43	63.7
15.2	III.3–III.7	—	—	5.86	0.2110	0.1618	0.0935	0.0099	0.0139	0.1335	0.1434	4.70	8.06	63.3
15.9	III.7–III.11	46.1	—	5.53	0.1990	0.1525	0.1025	0.0102	0.0119	0.1365	0.1467	5.13	7.80	68.6
15.9	II.18–III.11	11.3	+32.8	22.95	0.8261	0.6334	0.3748	0.0378	0.0521	0.5838	0.6216	4.58	8.23	70.7

The protein of the food, therefore, was utilized to the extent of 97.5 per cent, and the fat 96.2 per cent. The index of growth was 42.7 per cent showing that the fingerlings were growing at a favorable rate.

Owing to the accidental death of one of the animals, the experiment was renewed with the five remaining fingerlings, and continued for another 21 days. During this time the excreta were collected and analyzed five times. A short fast of 4 days intervened between the first and second parts of this experiment. The total weight of the five fingerlings was 34.8 gm., and they gained 11.3 gm. in the 21 days, or considering the average weight at the beginning and closing of the entire experiment, the fingerlings gained about 66 per cent in weight.

By reviewing the data pertaining to the second part of the experiment, certain differences will be observed as compared with the results obtained in the first part, which to a certain extent substantiate the experience gained in Experiment 000.5. In the first place, the ratio of dry feces to the dry matter of food is 50 per cent higher (cf. Table III, amount of food and of dry feces), and this increase in the fecal discharge runs parallel to the diminished utilization of the nutriment. The conclusion, therefore, is warranted that in this instance also the digestive activity was somewhat impaired by the short fast. The growth capacity, however, was not affected in this case, the fingerlings having grown more in the 3 weeks following the fast than during the 26 days preceding it. The gain in weight represented 49.8 per cent of the quantity of food per day and kilo, as compared with 42.7 of the earlier period.

Two things must be borne in mind in connection with these experiments: In both instances the evidence clearly indicates that fasting is deleterious to the digestive function of the trout. The difference in effect upon subsequent growth may probably be due to the fact that in Experiment 000.5, the intervening fast was a much more protracted one.

The last experiment on the effect of feeding raw beef heart was performed on the trout which had undergone a preliminary fast (F-1, Table I). In the course of 15 days of the experiment, this trout consumed 27.71 gm. of food with a content of 7.047 gm. of dry matter. Examining the last three columns of Table IV, one

TABLE IV.

Experiment 000.7. Raw Beef Heart.

Average temperature.	Period.	Initial or final weight.	Change of weight.	Amount of food.	N intake.	Fat intake.	Dry feces.	Nitrogen in feces.	Fat in feces.	Nitrogen in water (urine).	Total N eliminated.	N in feces in relation to N intake.	Fat in feces in relation to fat intake.	N in water in relation to N intake.
°C.		gm.	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
17.7	II.23–II.26	95.7	—	8.00	0.2880	0.2209	0.0460	0.0050	0.0355	0.1214	0.1264	1.74	16.08	42.2
14.1	II.26–III.1	—	—	3.56	0.1281	0.0983	0.1350	0.0128	0.0090	0.1168	0.1296	10.00	9.16	91.2
14.7	III.1–III.4	—	—	1.95	0.0702	0.0540	0.1280	0.0123	0.0265	0.0918	0.1041	17.52	49.10	130.8
15.9	III.4–III.7	—	—	5.40	0.1943	0.1490	0.1300	0.0113	0.0185	0.1326	0.1439	5.82	12.42	68.3
16.0	III.7–III.10	109.5	—	8.78	0.3162	0.2424	0.1920	0.0148	0.0341	0.1944	0.2092	4.68	14.07	61.5
15.7	II.23–III.10	13.8	+14.5	27.71	0.9968	0.7645	0.6310	0.0562	0.1236	0.6570	0.7132	5.64	16.16	65.9

notices the high proportion of fat in the feces, and the apparent retention of nitrogen as is shown by the low nitrogen content of excretions passed during the first several days after feeding was resumed. That the retention was purely temporary is seen from the fact that within the next few days the elimination was abnormally high, especially between the 6th and 9th days when it became actually 30.8 per cent more than the nitrogen contained in the food for that period. This delayed elimination of nitrogenous waste points to the possibility that the excretory mechanism of the trout suffered an injury in consequence of fasting, which it required several days, when food was given, to restore to normal functioning.

The similarity of the results obtained in these three experiments is very striking, those of Experiments 000.5 and 000.7 being practically identical.

The dry feces formed a much greater per cent of the dry matter consumed with the food than in any of the previous experiments (8 95 per cent, cf. Table VI). The utilization of the protein during the entire 15 day period is 94.4 per cent, which compares very favorably with the extent of utilization observed in the other experiments. The utilization of the fat which has been reduced to only 83.8 per cent demonstrates again, and more strikingly than in any of the previous experiments, the particularly deleterious influence of fasting upon fat utilization. The reason for this must be looked for in the longer duration of the fast. To explain these facts it may be necessary to assume that a more lasting damage was done to the glandular structures of the animal, the pancreas and the liver, which leads to a defective digestion and absorption of fats. Further investigation of this question would at any rate be desirable.

In spite of the low degree of utilization of the food materials, the index of growth was 50.1; in other words, half of the nutrient material fed has actually gone towards the building up of the body tissues. This result is of much significance, inasmuch as it adds further proof for the idea that neither the utilization of the food in digestion nor the actual quantity consumed determines the extent of the resulting growth of the organism. The trout in this experiment increased in weight at a greater rate than in any other of this series, while actually consuming the smallest quantity of food.

Feeding Cooked Beef Heart.

In the practical feeding of fish in hatcheries, the question is frequently asked, Shall the food be fed raw or cooked? The objection to cooked food on the ground that it is not the natural condition of nourishment of the fish, may well be dispensed with. Feeding fish in hatcheries is a problem in domestication, and the merits of a dietary system must be judged not by whether it resembles or deviates from the state of things in nature, that is wild nature, but by the results which can actually be achieved with it. To anticipate what will be brought out in the description of the following experiments, cooked food is neither more nor less utilized than the raw food, though possibly it has less growth-promoting quality. It is not as palatable to the fish as the raw food, and in my experience, the trout ate it much less willingly and in smaller amounts. In only one respect does feeding of cooked food present a decided advantage. On raw food the feces are gelatinous and fairly massive, while those resulting from cooked food are more or less dry and scanty. The feces therefore have no tendency to adhere to the sides of the aquaria, being in well formed compact masses; contamination of the water was never observed when cooked food was fed, and it remained remarkably clear for a number of days.

The food was put up as before, except that it was brought to a quick boil with a small volume of water, the fluid completely drawn off, and the meat packed in stock jars, refrigerated, and analyzed as usual.

Experiment 00.2 (Table V) was made on the same trout which served in Experiment 000.5, and the results of these two experiments are well suited for comparison. The animal was fed on the cooked beef heart for a considerable time to get it thoroughly accustomed to this food before the actual metabolic study was begun. The trout did not relish cooked food, and at best would eat only small quantities. In the 38 days which this experiment lasted, the trout consumed only 23.9 gm. of the food; the feces for this period contained 51 mg. of nitrogen and 34 mg. of fat. These two constituents of the diet were therefore utilized to the extent of 95.7 and 96.1 per cent respectively, which is very close to the values (96.1 and 94.2) which were found on raw meat.

TABLE V.

Cooked Beef Heart.

Experiment No.	Average temperature.	Period.	Initial or final weight.	Change of weight.	Amount of food.	N intake.	Fat intake.	Dry feces.	Nitrogen in feces.	Fat in feces.	N in water (urine).	Total N eliminated.	N in feces in relation to N intake.	Fat in feces in relation to fat intake.	N in water in relation to N intake.
	°C.		gm.	per cent	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent
00.2	16.7	IV.20–IV.30	100.6	—	3.5100	0.1776	0.1325	0.0330	—	—	0.1462	—	—	—	82.3
	16.3	IV.30–V.7	—	—	5.7150	0.2801	0.1982	0.0620	—	—	0.1859	—	—	—	66.4
	16.3	V.7–V.14	108.5	7.81	4.7500	0.2404	0.1793	0.0670	—	—	0.1809	—	—	—	75.3
	15.4	V.14–V.21	—	—	5.1750	0.2591	0.1875	0.0860	—	—	0.2069	—	—	—	79.9
	16.0	V.21–V.28	110.6	2.0	4.7600	0.2409	0.1796	0.0554	—	—	0.1841	—	—	—	76.4
	16.1	IV.20–V.28	10.0	+9.85	23.910	1.1981	0.8771	0.3034	0.0519	0.0340	0.9040	0.9559	4.33	3.88	75.5
00.3	16.2	IV.30–V.7	60.9	—	5.370	0.2534	0.1811	0.0420	—	—	0.1682	—	—	—	66.4
	16.3	V.7–V.14	66.6	9.02	5.120	0.2359	0.1746	0.0462	—	—	0.1804	—	—	—	76.5
	15.4	V.14–V.21	—	—	4.780	0.2415	0.1803	0.0874	—	—	0.1935	—	—	—	80.1
	16.0	V.21–V.28	67.4	1.2	4.960	0.2510	0.1871	0.0432	—	—	0.1839	—	—	—	73.3
	16.0	IV.30–V.28	6.5	+10.66	19.290	0.9818	0.7231	0.2188	0.0401	0.0283	0.7260	0.7661	4.09	3.91	74.0
00.4	16.2	IV.30–V.7	56.3	—	1.7500	0.0886	0.0660	0.0200	—	—	0.0699	—	—	—	78.9
	16.3	V.7–V.14	—	—	4.6450	0.2350	0.1753	0.0516	—	—	0.1449	—	—	—	61.7
	15.4	V.14–V.21	60.9	—	4.5850	0.2316	0.1728	0.0682	—	—	0.1583	—	—	—	68.4
	16.0	V.21–V.28	62.6	—	2.3800	0.1204	0.0898	0.0238	—	—	0.0999	—	—	—	82.9
	16.0	IV.30–V.28	6.3	+11.21	13.360	0.6756	0.5039	0.1636	0.0265	0.0378	0.4730	0.4995	3.92	7.50	70.1
	16.8	V.28–VI.1	62.6	—	3.150	0.1594	0.1188	0.0615	—	—	0.1097	—	—	—	68.9
	15.6	VI.1–VI.5	—	—	3.570	0.1806	0.1347	0.0690	—	—	0.1230	—	—	—	68.1
	17.8	VI.5–VI.9	64.8	—	3.600	0.1822	0.1358	0.0450	—	—	0.1419	—	—	—	77.9
	16.5	VI.9–VI.13	67.8	—	4.010	0.2029	0.1513	0.0475	—	—	0.1074	—	—	—	52.9
	17.4	VI.13–VI.17	69.1	—	2.730	0.1381	0.1030	0.0688	—	—	0.1359	—	—	—	98.4
	16.8	V.28–VI.17	6.5	+8.31	17.060	0.8632	0.6436	0.2918	0.0763	0.0479	0.6179	0.6942	8.84	7.45	71.6
	16.4	IV.30–VI.17	12.8	+22.74	30.421	1.5308	1.1475	0.4554	0.1028	0.0857	1.0909	1.1937	6.68	7.47	70.9

The per cent of nitrogen and fat in the dry feces is much greater than on a diet of raw meat, and this condition is very general as can be seen by comparing the data in Columns 5 and 6 of Table VI. This very high percentage, however, is of no significance, being merely due to the fact that the feces are more compact and dry when the trout are kept on the cooked meat régime. It is more significant that the amount of dry feces presents the same proportion of the dry matter of the consumed food (3 to 4 per cent) no matter whether the food has been cooked or not.

TABLE VI.

Experiment No.	Duration.	Nature of food.	Ratio of dry feces to dry matter of food.	Dry feces.		Utilization.		Average intervals between feedings.	Food consumed	Increase in body weight.	Index of growth.
				Nitrogen.	Fat.	Protein.	Fat.		Per kg. and per 24 hrs.		
	days		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*hrs.*	*gm.*	*gm.*	
000.5(a)	32	Raw beef heart.	4.99	10.0	10.0	96.1	94.2	48	37.5	17.40	46.5
000.5(b)	15	"	7.80	9.6	9.3	94.8	91.7	48	26.7	9.73	36.5
000.6(a)	26	"	3.52	9.1	6.4	97.5	96.2	48	25.0	10.67	42.7
000.6(b)	21	"	6.42	10.0	10.0	95.4	91.8	48	27.0	13.45	49.8
000.7	15	"	8.95	8.9	17.9	94.4	83.8	48	18.0	17.02	50.1
00.2	38	Cooked beef heart.	3.4	19.1	12.7	95.7	96.1	41	6.0	2.50	41.7
00.3	28	"	3.0	20.9	14.6	95.9	96.1	34	10.78	3.63	33.7
00.4	48	"	3.7	22.6	18.8	95.7	92.5	36	10.11	4.25	42.0

The results of Experiment 00.3 (Table V) are essentially the same, though this trout has shown a somewhat smaller increase in weight. Experiment 00.4 which lasted 48 days was made on a trout which had been fasting a week before the experiment commenced. The protein of the food was utilized as usual—95.7 per cent, but only 92.5 per cent of the fat was utilized. This further confirms the point emphasized already in this paper, of the defective utilization of fat by trout that have undergone even a brief fast. The amount of food this trout ingested per day and kilo of body weight was 10.11 gm. The daily increment in

weight per kilo was 4.25 gm.; in other words, the growth index was 42. When we compare the relative values of cooked and raw meat as the diet for trout, we can indicate the following advantages of the latter: its greater palatability and greater growth-promoting quality. This can be seen at once when the average growth index of Experiments 000.5 to 000.7, which is 45, is compared with the same of Experiments 00.2 to 00.4, which is only 39. This might perhaps be objected to on the ground that the difference in the body weight increments is due rather to the great difference in the quantity of food which the trout has consumed in these two series of experiments. It must be recalled, however, that the growth index does not furnish information as to the actual or absolute increment in body weight, but indicates the fraction of the nutrient material which has become permanently incorporated as a part of the organism. The two kinds of food have been apparently ample in amount to insure not only maintenance, but a further increase in weight, but in the case of the cooked beef heart a smaller relative increase in body weight was secured. It is possible that the lesser effect of the latter in producing growth was due to the fact that in the process of boiling, some of its water-soluble growth-stimulating substances have been dissolved out. The fact that the food which has been boiled and thus lost a large part of its water content was as well utilized by the trout as raw meat, suggests that it would be practicable to utilize thoroughly desiccated food as trout diet. The food soaked in water before feeding would probably retain its gustatory quality, as well as its growth-promoting quality. This might be an excellent method for preparing on a large scale and distributing the food for trout hatcheries. Experiments, which were planned with a view of studying the metabolic value of such desiccated foods, remained unperformed owing to the unexpected interruption of the investigation.

Feeding Beef Liver.

Beef or pig liver is one of the staple diets in trout hatcheries, and a series of experiments was started to study its nutritive value. The liver was freed from all blood vessels and ducts, and the parenchyma alone was frozen solid and ground in a meat

grinder to a fine pulp. The food was then kept in the refrigerator and analyzed as usual. Unfortunately it was impossible to feed this food directly from forceps as was done with the beef heart. It was necessary instead to throw a quantity of food into the aquaria and leave it there for half an hour. The trout was then removed to another aquarium with fresh water, in which the excreta were collected, while the unconsumed food was collected over glass wool in a large filtering tumbler. The filtrate was made up to a definite volume, of which aliquot portions were used to determine the nitrogen, fat, and sugar that were washed out from the food by the water. The solid residue was dried at 100°, powdered, and the composite sample for the entire experimental period was analyzed in the usual way.

Knowing the quantity of food thrown into the aquarium, the amounts of nitrogen, fat, and carbohydrate were computed from the analytical data pertaining to the particular food sample. From this were subtracted the amounts of nitrogen, fat, and carbohydrate, found both in the water and in the solid residue. The consumed amount of each constituent was then determined by the difference.

In his study of the digestion of fish, Knauthe[1] made use of this method exclusively. Apart from the disadvantage which it shares with all indirect methods, the method is time-consuming and presents so many unforeseen difficulties as to render the results at times of no value.

In the matter of recovering the fat, Knauthe's procedure, though very simple, consisting merely in extracting an aliquot portion of the water with ether, on closer examination proves of little or no value. Knauthe has not performed blank experiments to determine how closely the recovered amount corresponded with that washed out by the water; he therefore had no occasion to be apprehensive about the acceptability of his results.

As was pointed out above, I considered the performance of blank experiments essential, and have relied on these in deciding whether or not the analytical procedure in use was adequate. By following Knauthe's method, I found that the results were so widely different from the expected values as to make them worthless.

[1] Knauthe, K., *Z. Fischerei*, vi, 1898, 139.

It will be noted further that in all the experiments the animals were allowed to remain in the feed jars half an hour, after which they were removed to fresh water. Knauthe on the other hand, allowed his animals to remain in the aquaria containing food for a long time, and separated mechanically the feces from the solid food particles. It need hardly be pointed out that such a procedure is too crude to warrant great confidence in the significance of experimental results. The bacterial growth in suspensions of nutrient material would be sufficient to vitiate the results. In a number of blank experiments a weighed quantity of liver pulp was put in the aquaria (without trout), left there half an hour as usual, then the solid residue and the filtered water were analyzed. In the case of the nitrogenous material and the sugar, the recovery was complete within less than 1 per cent, but in the matter of the fat, as determined by direct extraction of the water with ether, the values were usually so low as to render the data of no importance. The need of a different and better method was keenly appreciated, and attempts in this direction were started. The main difficulty to be remembered, was that a relatively small quantity of fat had to be recovered quantitatively from a large bulk of water.

All the determinations were in duplicate. The nitrogen was determined by the Kjeldahl method, and one-tenth portions of the entire water were used for the analysis. The sugar was determined by Bertrand's method in one-quarter portions. The sugar analysis was made on the perfectly clear filtrate, which was obtained by treating the water with aluminum cream to free it of the protein. On extracting this clear filtrate with ether, I invariably found that there was no fat present. This suggested a method of determining the fat which is evidently carried down with the aluminum precipitate. By drying this voluminous gelatinous precipitate, powdering it, and extracting it with ether, it was hoped that accurate estimate of the fat suspended in the water would be obtained. While this method, or some similar modification of it, has good possibilities, a number of difficulties were encountered in its practical application, which were not entirely removed before this work was interrupted, and the matter of utilization of the fat from liver was not touched in this investigation.

TABLE VII.

Raw Beef Liver.

Experiment No.	Average temperature.	Period.	Initial and final weight	Change in weight.	Amount of food.	N intake.	Glucose intake.	N in feces.	Fat in feces.	Sugar in feces.	N in water (urine).	Total N eliminated.	N in feces in relation to N intake.	N in water in relation to N intake.	Sugar in feces in relation to sugar intake.
	°C.	*days*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
02 (a)	14	14	35.8 38.1	+ 6.4	23.580	0.2470	0.3285	0.0235	0.0540	0.0432	0.1520	0.1755	9.5	61.5	13.1
03	16	21	59.1 50.6	−14.4	84.830	0.6114	0.4100	0.1005	0.1246	0.0127	0.4413	0.5418	16.4	72.1	3.1
03 (a)	16.5	13	49.4 50.3	+ 1.8	51.010	0.2750	0.1295	0.0420	0.0704	0.0125	0.2530	0.2950	15.3	92.0	9.7

The feeding of liver has not met with success in my hands. A number of circumstances have probably conspired to make these experiments less definite than those of the preceding series. Many experiments failed, owing to the death or ill condition of the trout. Three experiments are recorded in Table VII, and these will perhaps throw light on the question of liver as a trout food. It will be seen that the trout did not grow as well as those fed on beef heart; indeed, one specimen lost 8.5 gm. The utilization of protein (nitrogen) was very low (90.5 to 83.6 per cent). The utilization of glucose likewise shows wide fluctuation, from 96.9 to 86.9 per cent; that of the fat ranged about 90 per cent, but as the analytical data were not entirely reliable, these results have not been included in the table.

Before concluding, one other point should be mentioned. In the course of the half hour during which the liver pulp remained in the aquaria, as much as 60 to 75 per cent of its nitrogenous material has been dissolved by the water. It is a conservative estimate that 50 per cent of the fat was washed out, and none of the glucose was left in the residue, the glucose having been entirely dissolved out by the water. This happened in spite of the fact that the food was contained in a limited quantity of water, agitated only by the swimming movements of the trout.

In hatcheries where the trout are kept in rapid streams of water, it is questionable whether a food, which will so easily give up its components to the water, is a particularly valuable diet. Its inferiority to beef heart as a dietary article for trout is amply demonstrated by the results of the experiments recorded here.

SUMMARY.

A study of the utilization of the fundamental food materials—protein, fat, and carbohydrate—has been made on trout. The per cent of utilization under normal circumstances was found to be high. Fasting has a deleterious effect on absorption, particularly of the fat.

Fasting trout eliminate 80 to 90 mg. of nitrogen per day and per kilo. But if the fast has been preceded by a liberal diet, the nitrogen elimination for the first few days may greatly exceed

this. About one-fifth of the loss in body weight is at the expense of protein.

A comparison of diets consisting of raw and cooked beef heart showed that both are equally well utilized in the organism of the trout, but a greater gain in body weight is secured from the former. Increase in weight does not bear a direct relation to the absolute quantity of food consumed.

This study fails to justify the use of beef liver as a dietary article for trout.

ANIMAL CALORIMETRY.

FIFTEENTH PAPER.

FURTHER EXPERIMENTS RELATIVE TO THE CAUSE OF THE SPECIFIC DYNAMIC ACTION OF PROTEIN.

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INTRODUCTION.

The work accomplished in this laboratory[1] has shown that certain amino-acids, such as glycocoll and alanine, when given to a dog cause a great increase in the heat production of the animal, and that this increase in heat production is proportional to the number of hydroxy-acid molecules, glycollic or lactic, which can be liberated on the deamination of glycocoll and alanine. Since no increase in metabolism was caused by the ingestion of glutamic acid, it was concluded that the processes of deamination or of urea formation played no rôle in the increased heat production. The proof that amino-acids themselves are not stimuli of metabolism lies in the fact that that quota of ingested protein which is absorbed as amino-acids and rebuilt into new body pro-

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[1] Lusk, G., *J. Biol. Chem.*, 1912–13, xiii, 155; 1915, xx, 555.

tein exerts no specific dynamic action; and the proof that the increased heat production does not come from the oxidation of the materials themselves is demonstrated by the observation that when glycocoll and alanine are given in phlorhizin glycosuria they are transformed into glucose without oxidation, are eliminated in the urine, and yet exert a stimulating effect on the metabolism.

In 1915 Grafe[2] published a series of experiments upon rabbits, dogs, and men relative to the specific dynamic action of protein, some of which experiments, especially those concerning glycocoll and alanine, confirmed, while others contradicted Lusk's researches, and yet others were self-contradictory. For example, Grafe found in a dog that the absorption of oxygen was increased between 16 and 17 per cent during $3\frac{1}{3}$ hours following the ingestion of 50 gm. of glutamic acid (as a sodium salt), whereas Lusk had found no increase after giving 20 gm. Furthermore, Grafe reported that after giving 7.5 gm. of asparagine to a man the metabolism was uninfluenced during successive periods, respectively, of $4\frac{1}{2}$, 4, and 2 hours duration, although he found an increase of 11 per cent in the oxygen absorption after giving 47 gm. to another man. This last experiment is open to the following criticisms: (1) The man was in the hospital undergoing treatment for syphilis. (2) The basal metabolism was determined 5 days before the day of asparagine ingestion, during which interval the patient is stated to have increased in weight from 50.2 to 51.5 kilos. (3) The respiratory quotient of the 10 hour interval of the basal metabolism period was 0.95, notwithstanding the fact that this period began 12 hours after taking food. Such a respiratory quotient could scarcely have been obtained had the patient been given sugar in large excess on the day previous.[3] This leads to the suspicion that the oxygen determination was too low. The carbon dioxide excretion rose only 5 per cent as a result of the asparagine ingestion.

This questionable determination of the basal metabolism throws doubt upon reported increases in the metabolism of this man, of 13 per cent after giving 28 gm. of phenylalanine; of 14 per cent

[2] Grafe, E., *Deutsch. Arch. klin. Med.*, 1915–16, cxviii, 1.

[3] Benedict, F. G., Emmes, L. E., and Riche, J. A., *Am. J. Physiol.*, 1910–11, xxvii, 383.

after giving 12 gm. of ammonium chloride; and of 22 per cent after giving 42 gm. of acetamide.

On the basis of these and other experiments Grafe comes to the conclusion that the specific dynamic action of protein is largely due to the liberation of the NH_2 radicle of amino-acids. His table showing this relation in man is inconclusive. However, the table showing the relation between nitrogen intake and extra heat production in the dog may be quoted in part as follows:

Substance given.	N	Amount in food.	N content.	Increase in calories per gm. of N ingested. $\frac{\text{Calories}}{\text{N}}$	Author.
	per cent	*gm.*	*gm.*		
Glycocoll	18.7	25	4.66	4.48	Lusk.
Alanine	15.7	20	3.14	4.00	"
"		50 (?)	7.85 (?)	4.5 (?)	Grafe.
Leucine	10.7	20	2.14	2.9	Lusk.
Glutamic acid	9.5	20	1.90	0	"
" "		50 (?)	4.75 (?)	5.6 (?)	Grafe.
Tyrosine	7.7	20	1.55	5.0	Lusk.
100 gm. meat	3.0	100	3.00	4.8	"

The interrogation points after Grafe's work, which are found in the above tabulation, are in his original table and are due to the fact that the materials given to the dog were in small part vomited.

It is evident that the main disagreement in the results obtained in the two laboratories lies in the difference in the outcome of the two experiments in which glutamic acid was ingested. The validity of Lusk's conclusions has also been challenged by others[4] and it seemed desirable to subject the matter to further investigation. As no glutamic acid was available, aspartic acid was employed in its stead. Asparagine was also given. Since this latter substance and glycocoll both contain 18.7 per cent of nitrogen, it would follow from Grafe's hypothesis that each should exert the same specific dynamic action. Also, the theoretically interesting administration of acetamide was worthy of

[4] Notably by Dr. H. H. Mitchell in private correspondence.

repetition and, in addition to this, experiments were instituted on the ingestion of succinic acid, which Ringer[5] and coworkers deem to be an intermediary metabolite of glutamic acid. *The following experiments wholly failed to substantiate Grafe's conclusions.*

EXPERIMENTAL.

The same experimental procedure was employed as in the work described in former papers.[6] The ingestion of materials followed about 18 hours after giving the usual "standard diet."

Urinary Nitrogen.

Dog XVI.—After giving 10 gm. of aspartic acid dissolved in hydrochloric acid, the nitrogen excretion during the first 3 hours and 37 minutes amounted to 0.25 gm. per hour in contrast with not more than 0.1 gm. per hour during periods of the determination of the basal metabolism. The administration of *hydrochloric acid* alone slightly increased the nitrogen elimination above the basal level to 0.12 gm. per hour from 0.09 gm. per hour.

Dog XVII.—The more striking details of the urinary analyses in this dog may be presented in Table I. The results after the ingestion of materials were obtained from the urine of the first 3 to 4 hours thereafter.

It is evident from Table I that *asparagine* and *ammonium citrate* were both freely absorbed and converted into urea, whereas *acetamide*, though absorbed, did not increase the urea in the urine. The ingestion of *succinic acid* increased the total nitrogen in the urine as did hydrochloric acid in Dog XVI.

Computation of the Respiratory Metabolism.—A method for the computation of the metabolism of ingested glycocoll has been described in a previous paper of this series.[6] It was based on the fact that when glycocoll is given in phlorhizin glycosuria 22 per cent of it is eliminated per hour as extra sugar in the urine during the 2nd and 3rd hours and a lesser quantity thereafter. This was accepted as a better guide to the rapidity of the normal me-

[5] Ringer, A. I., Frankel, E. M., and Jonas, L., *J. Biol. Chem.*, 1913, xiv, 539.

[6] Lusk, *J. Biol. Chem.*, 1915, xx, 555.

tabolism than the nitrogen excretion of the corresponding hours, since this excretion is known to lag considerably behind the formation of urea in the body.

In the present series of experiments it has been arbitrarily assumed that 50 per cent of the ingested aspartic acid, asparagine, and succinic acid were metabolized during the 2 hour period which included the 2nd and 3rd hours after the ingestion of the

TABLE I.

Date.	Experiment No.	Food.	Total N.	Urea N.	NH_3N
1918			*gm.*	*gm.*	*gm.*
Feb. 11	16	Basal.	0.130		
" 12	17	Asparagine, 15 gm.	0.170	0.157	0.010
" 18	21	" 15 "	0.182	0.167	0.011
" 21	23	Lard, 25 gm.	0.117	0.101	0.010
" 26	25	Asparagine, 15 gm.	0.219	0.207	0.009
" 28	26	Basal.	0.115		
Mar. 4	28	"	0.106		
" 7	29	Succinic acid, 11.2 gm.	0.140		
" 8	30	Basal.	0.101		
" 9	31	Succinic acid, 11.2 gm.	0.147		
" 12	32	Basal.	0.097		
" 14	34	Acetamide, 12 gm.	0.190	0.070	0.020
" 15	35	Basal.	0.135		
Jan. 16	5	Acetamide, 12 gm.	0.180	0.064	0.016
" 17	6	Basal.	0.093		
" 25	11	"	0.110		
" 29	12	Ammonium citrate, 6 gm.	0.253	0.179	0.025

substances. The computations were based on the following considerations.

Aspartic Acid.—Calories per gm. = 2,882. The oxidative physiological reaction:

$$2\ C_4H_7NO_4 + 6\ O_2 = 7\ CO_2 + 5H_2O + CN_2H_4O$$
$$1\ gm. + 0.721\ gm. = 1.157\ gm. + 0.225\ gm.$$
$$2{,}882\ cals. = 2{,}312\ cals. + 570\ cals.$$

Physiological values: R. Q. = 1.17, 1 gm. = 2,312 calories, 1 liter O_2 = 4.578 calories.

Asparagine.—Calories per gm. = 3,065. The oxidative physiological reaction:

$$C_4H_8N_2O_3.H_2O + 3\ O_2 = 3CO_2 + 3H_2O + CN_2H_4O$$
$$1\ gm. + 0.64\ gm. = 0.88\ gm. + 0.4\ gm.$$
$$3{,}065\ cals. = 2{,}054\ cals. + 1{,}011\ cals.$$

Physiological values: R. Q. = 1. 1 gm. = 2,054 calories, 1 liter O_2 = 4.562 calories.

Succinic Acid.—Calories per gm. = 3,026. The oxidative physiological reaction:

$$2C_4H_6O_4 + 7\ O_2 = 8\ CO_2 + 6\ H_2O$$
$$1\text{ gm.} + 0.95\text{ gm.} = 1.49\text{ gm.}$$

Physiological values: R. Q. = 1.14, 1 liter O_2 = 4.550 calories.

Ammonium Citrate.—Calories unknown. The oxidative physiological reaction:

$$2\ C_6H_{17}N_3O_2 + 9\ O_2 = 9\ CO_2 + H_2O + 3\ CN_2H_4O$$
$$1\text{ gm.} + 0.598\text{ gm.} = 0.815\text{ gm.} + 0.370\text{ gm.}$$

Physiological value of R. Q. is unity.

Influence of Aspartic Acid and of Hydrochloric Acid.

10 gm. of aspartic acid dissolved in 200 cc. of a solution containing 0.4 per cent of hydrochloric acid and 0.2 gm. of Liebig's extract of beef were warmed to a temperature of 50°C. and administered to a dog through a stomach sound. The results were compared with those obtained when a like amount of hydrochloric acid was given, and also with the *basal metabolism* when no food was given.

The results may be summarized in Table II, which shows the influence on the heat production of Dog XVI of 10 gm. of aspartic acid in 200 cc. of 0.4 per cent HCl solution and of the latter alone. Results are in 2 hour periods which began 1 hour after the ingestion of the material.

TABLE II.

Date.	Experiment No.	Food.	R. Q.	Calories.	
				Indirect.	Direct.
1917					
Nov. 12	10	Basal.	0.78	40.67	40.65
" 14	11	HCl.	0.78	42.48	41.38
" 19	12	Aspartic acid + HCl.	0.85	42.49	40.54
" 21	13	Basal.	0.76	38.94	38.84
" 23	14	"	0.72	41.28	40.89
" 26	15	Aspartic acid + HCl.	0.87	42.05	39.22
" 27	16	Basal.	0.76	40.23	37.36
" 28	17	HCl.	0.80	20.13*	39.46
Dec. 5	18	Basal.	0.78	38.78	37.40

* 1 hour only.

The administration of another dose of hydrochloric acid resulted in the death of the animal, with all symptoms of acid poisoning, a regrettable accident, since the observation on Nov. 28 was incomplete on account of a slight leak of oxygen during the 2nd hour.

The higher respiratory quotients on the days of aspartic acid ingestion conform with expectations.

The evidence found in Table II may be further summarized as follows so as to indicate the influence of the two factors, aspartic acid and hydrochloric acid:

No. of experiments.	Food.	Calories.			
		Indirect.	Increase above basal.	Direct.	Increase above basal.
5	Basal.	40.0		39.0	
2	HCl.	42.5*	2.5	40.4	1.4
2	Aspartic acid + HCl.	42.7	2.7	40.0	1.0

* One experiment only.

It is evident from this tabulation that, *although the administration of hydrochloric acid may cause a slight increase of 2.5 calories (6 per cent) in the basal metabolism, the simultaneous administration of 10 gm. of aspartic acid will cause no further increase.*

If one compares these results with computations of the first 2 hours of the recorded metabolism after giving 5.5 gm. of glycocoll to Dog III,[7] one arrives at the following table:

Dog.	Experiment No.	Food.	N in food.	Calories.	
				Indirect.	Increase.
			gm.		
III	5, 6, 21	Basal.		39.6	
	13	Glycocoll, 5.5 gm.	1.03	43.3	3.7
	9, 10, 14	Alanine, 5.5 gm.	0.87	43.0	3.4
XVI	11	HCl.		42.5	
	12, 15	Aspartic acid, 10 gm., + HCl.	1.05	42.7	0.2

These experiments with aspartic acid corroborate the former work with glutamic acid and indicate that the two dibasic acids occurring

[7] Lusk, *J. Biol. Chem.*, 1915, xx, 568.

in protein exert no specific dynamic action upon metabolism. The increase of 17 per cent in the metabolism of a dog during the first $3\frac{1}{3}$ hours after giving 50 gm. of glutamic acid, as observed by Grafe, might be due to the large number of metabolites introduced into the circulation—just as 50 gm. of glucose would effect the same result.

Influence of Asparagine.

Owing to the insolubility of asparagine, it was given mixed with 25 gm. of fat. When glutamic acid was given with lard to a phlorhizinized dog[8] the former was absorbed and converted into glucose within 12 hours after its administration, and this

TABLE III.

Date.	Experiment No.	Food.	R.Q.	N in urine.	Calories.	
					Indirect.	Direct.
1918				*gm.*		
Jan. 25	11	Basal.	0.83	0.220	43.09	37.53
Feb. 11	16	Lard, 25 gm.	0.82	0.260	45.78	
" 12	17	" 25 " + asparagine, 15 gm.	0.92	0.340	42.20	44.39
" 18	21	" 25 " + " 15 "	0.88	0.364	45.06	43.59
" 21	23	" 25 "	0.80	0.234	45.29	42.16
" 26	25	" 25 " + asparagine, 15 gm.	0.88	0.438	45.31	42.19
" 28	26	Basal.	0.83	0.230	43.09	38.60

older work suggested the present procedure. In the present series of experiments evidences of the absorption of asparagine are found in the increased quantity of urinary nitrogen and in the consistently higher respiratory quotients obtained on the days of ingestion of asparagine, which substance gives a respiratory quotient of unity.

Table III presents the main results of the investigation of the influence on the heat production of Dog XVII of 15 gm. of asparagine given with 25 gm. of lard, and of the latter alone. Results are in 2 hour periods which began 1 hour after the ingestion of the substance.

[8] Lusk, *Am. J. Physiol.*, 1908, xxii, 174.

It is evident from Table III that the addition of 15 gm. of asparagine containing 2.8 gm. of nitrogen to a diet containing a small amount of fat has no effect whatever upon the heat production of a dog. It was established by Murlin and Lusk[9] that when glycocoll and fat are absorbed together the increase in the heat production amounts to the sum of the increases which either substance alone would have produced. The increase in the metabolism of a dog during the 2nd and 3rd hours after the ingestion of 20 gm. and of 10 gm. of glycocoll, as determined by Lusk,[7] may thus be summarized:

Dog III.

Glycocoll.	N in food.	Increase in metabolism during 2 hrs.
gm.	*gm.*	*calories*
10	1.9	7.6
10	1.9	8.8
20	3.7	14.6
20	3.7	13.8

It is evident, therefore, that asparagine and glycocoll, which contain the same percentage quantity of nitrogen, behave very differently in metabolism, the first being without specific dynamic action and the other exerting the most powerful specific dynamic action of any of the amino-acids contained in protein which have thus far been tested. Therefore, the specific dynamic action of protein is not due to the NH_2 radicle of the amino-acid molecule.

Influence of Succinic Acid.

If the dibasic acid, glutamic acid, is oxidized at the α-amino carbon position with cleavage of carbon dioxide, succinic acid would result. If in accordance with Ringer's[5] conception, this is the first intermediary product of the metabolism of glutamic acid, then the ingestion of this substance should show no pronounced effect upon the metabolism. That such is the case is borne out in Table IV, which shows the influence of 11.8 gm. of succinic acid dissolved in 200 cc. of water upon the heat production of Dog XVII, periods of 2 hours.

[9] Murlin, J. R., and Lusk, G., *J. Biol. Chem.*, 1915, xxii, 15.

It is evident that 11.8 gm. of succinic acid, which is the amount that might have been derived from 14.7 gm. of glutamic acid, has no appreciable influence upon the metabolism of a dog.

Since succinic acid would produce a respiratory quotient of 1.14, higher quotients are observed on the days of its ingestion than on the days of basal metabolism.

TABLE IV.

Date.	Experiment No.	Food.	R. Q.	N in urine.	Calories.	
					Indirect.	Direct.
1918				*gm.*		
Feb. 28	26	Basal.	0.83	0.230	43.29	38.60†
Mar. 4	28	"	0.81	0.212	43.83	39.74†
" 7	29	Succinic acid, 11.8 gm.	0.87	0.280	43.98	44.28
" 8	30	Basal.	0.81	0.202	41.65	37.86†
" 9	31	Succinic acid, 11.8 gm.	0.93	0.147*	21.07*	20.60*
" 12	32	Basal.	0.80	0.194	41.21	39.48

* 1 hour only.

† First hour low. It is probable that the divergence between the indirect and the direct calorimetry, was due to the fact that the iron work of the calorimeter was not fully warmed at the beginning of the experiment. The room was allowed to get cold each night.

Influence of Acetamide, 12 Gm.

Acetamide is a chemical isomer of glycocoll and, according to Grafe, is deaminized after ingestion by a man and increases the heat production. In the dog it is neither deaminized nor does it increase the heat production. The latter fact appears from Table V, which shows the effect of 12 gm. of acetamide (= 2.04 gm. N) dissolved in 200 cc. of water upon the heat production of Dog XVII. Results are in 2 hour periods which began 1 hour after the ingestion of the substance.

TABLE V.

Date.	Experiment No.	Food.	R. Q.	N in urine.	Calories.	
					Indirect.	Direct.
1918				*gm.*		
Mar. 12	32	Basal.	0.80	0.194	41.21	39.48
" 14	34	Acetamide, 12 gm.	0.87	0.380	39.83	38.03
" 15	35	Basal.	0.88	0.270	40.82	

Influence of Citrate of Ammonium, 6 Gm.

When 6 gm. of citrate of ammonium, containing 1.04 gm. of nitrogen, were given to the dog in one experiment there was no appreciable increase in the metabolism. As the caloric value of the substance was unknown, the exact heat production could not be estimated, but using the ordinary procedure, the indirect heat production for 2 hours may be calculated as follows:

Date.	Experiment No.	Food.	Calories.
1918			
Jan. 25	11	Basal.	43.09
" 29	12	Ammonium citrate, 6 gm.	44.76

This rise of 1.65 calories (which is within the limits of error in the determination of the basal metabolism of a dog) may be compared with one of 3.7 calories already cited as having been produced after giving 5.5 gm. of glycocoll containing 1.03 gm. of nitrogen.

SUMMARY.

It has been shown that 200 cc. of 0.4 per cent of *hydrochloric acid* may slightly increase the metabolism of a dog; that *aspartic acid* and *asparagine* exert no specific dynamic action upon metabolism in the sense that glycocoll and alanine do; that *succinic acid* (a possible intermediary metabolite of glutamic acid) is without the power to increase the heat production, which accords with the previously determined behavior of glutamic acid; that *acetamide* is not deaminized by the dog nor does it increase the heat production of the animal.

The conclusion formerly expressed that the processes of deamination and urea formation have nothing to do with the specific dynamic action of protein is upheld by these later researches.

Some assistance was given in accomplishing this work by second year medical students: Messrs. T. M. French, H. B. Adams, M. M. Harris, A. G. Davidson, W. I. Galland, and H. E. Montero.

TABLE VI.—*General*

Date.	Experiment No.	Time.	CO_2	O_2	R.Q.	H_2O	Urine, basal N.	Non-protein R.Q.	Calories.		
									Protein.	Amino-acid.	Non-protein.
1917		*p.m.*	*gm.*	*gm.*		*gm.*	*gm.*				
Nov. 12	10	2.40–4.40	13.13	12.25	0.78	21.12	0.168	0.78	4.46		36.21
" 14	11	2.56–4.56	13.81	12.83	0.78	20.79	0.228	0.80	6.04		36.44
" 19	12	3.11–5.11	15.37	13.06	0.86	21.45	(0.168)	0.72	4.46	11.56	26.47
" 21	13	2.36–4.36	12.40	11.79	0.76	19.39	0.166	0.76	4.24		34.70
" 23	14	2.33–4.33	12.59	12.65	0.72	24.94	0.200	0.71	5.30		35.98
" 26	15	3.06–5.06	15.75	13.12	0.87	18.98	(0.200)	0.75	5.30	11.56	25.99
" 27	16	2.32–4.32	12.71	12.21	0.76	15.29	0.192	0.75	5.10		35.13
" 28	17	3.10–4.10	6.67	6.06	0.80	9.94	0.123	0.80	3.26		16.87
Dec. 5	18	2.03–4.03	12.61	11.68	0.79	18.47	0.180	0.78	4.78		34.00

TABLE VII.—*General*

Date.	Experiment No.	Time.	CO_2	O_2	R.Q.	H_2O	Urine, basal N.	Non-protein R.Q.	Calories.		
									Protein.	Special substance.	Non-protein.
1918		*p.m.*	*gm.*	*gm.*		*gm.*	*gm.*				
Jan. 25	11	2.00–4.00	14.63	12.86	0.83	17.26	0.220	0.83	5.84		37.25
" 29	12	2.08–4.08	15.82	13.20	0.87	19.20	(0.220)*	0.88	5.84		38.92
Feb. 11	16	3.03–4.03	15.37	13.71	0.82	17.03	0.260	0.82	6.90		38.88
" 12	17	1.16–3.16	16.07	12.80	0.92	18.59	(0.260)	0.88	6.90	15.40	19.90
" 18	21	2.22–4.22	16.58	13.71	0.88	17.58	(0.110)	0.82	5.84	15.40	23.82
" 21	23	1.49–3.49	15.01	13.60	0.80	15.18	0.234	0.80	6.20		39.09
" 25	25	1.25–3.25	16.73	13.78	0.88	17.33	(0.234)	0.83	6.20	15.40	23.71
" 28	26	1.19–3.19	14.78	12.90	0.83	17.00	0.230	0.84	6.10		37.19
Mar. 4	28	12.52–2.52	14.64	13.13	0.81	16.38	0.212	0.82	5.62		38.21
" 7	29	12.29–2.29	16.99	13.67	0.90	23.85	0.280	0.71	7.42	17.85	18.71
" 8	30	12.36–2.36	13.93	12.47	0.81	16.43	0.202	0.81	5.36		36.29
" 9	31	12.13–1.13	8.27	6.54	0.95	9.06	(0.147)	0.73	3.90	8.92	8.25
" 12	32	12.40–2.40	13.63	12.37	0.80	16.76	0.194	0.80	5.14		36.07
" 14	34	12.23–2.23	14.00	11.76	0.87	17.43	(0.194)	0.87	5.14		34.69
" 15	35	12.13–2.13	14.56	12.07	0.88	16.18	0.135	0.89	7.16		33.66

* Nitrogen values in parentheses are assumed from nearby records of the basal

Summary of Dog XVI.

Calories.		Body temperature.			Morning weight.	Behavior of dog.	Food.
Total calculated.	Total found.	Start.	End.	Difference.			
		°C.	°C.	°C.	kg.		
40.67	40.65	38.48	38.29	−0.19	10.5	Quiet.	Basal.
42.48	41.38	38.78	38.47	−0.31	10.8	"	2 p.m., 200 cc. 0.4 per cent HCl.
42.49	40.54	38.47	38.26	−0.21	10.8	"	2.20 p.m., 10 gm. aspartic acid + 200 cc. 0.4 per cent HCl + 0.2 gm. meat extract.
38.94	38.84	38.56	38.45	−0.11	10.6	"	Basal.
41.28	40.89	38.60	38.36	−0.24	10.7	"	"
42.85	39.22	38.51	38.30	−0.21	10.9	"	2.10 p.m., aspartic acid as above.
40.23	37.36	38.67	38.54	−0.13	10.7	"	Basal.
20.13	18.34	38.69	38.49	−0.20	10.7	"	2 p.m., 200 cc. 0.4 per cent HCl.
38.78	37.40	38.35	38.45	−0.10	10.9	"	Basal.

Summary of Dog XVII.

Calories.		Body temperature.			Morning weight.	Behavior of dog.	Food.
Total calculated.	Total found.	Start.	End.	Difference.			
		°C.	°C.	°C.	kg.		
43.09	37.53	38.35	38.17	−0.18	14.7	Quiet.	Basal.
44.76	44.24	38.44	38.37	−0.07	14.9	"	12 m., 12.30, and 1 p.m., in 3 portions, 6 gm. ammonium citrate in 200 cc. water.
45.78					14.7	"	1 p.m., 25 gm. lard.
42.20	44.39	38.38	38.43	+0.05	14.7	"	12.35 p.m., 15 gm. asparagine + 25 gm. lard.
45.06	43.59	38.51	38.45	−0.06	14.8	"	1.35 p.m., asparagine as above.
45.29	42.16	38.28	38.34	+0.06	14.8	"	1.10 p.m., 25 gm. lard.
45.31	42.19	38.30	38.36	+0.06	14.8	"	12.35 p.m., asparagine as above.
43.09	38.60	38.30	38.24	−0.06	14.8	"	Basal.
43.83	39.74	38.44	38.27	−0.17	14.9	"	"
43.98	44.28	38.58	38.36	−0.22	15.1	"	11.40 a.m., 11.8 gm. succinic acid in 200 cc. water at 39°C.
41.65	37.86	38.30	38.09	−0.21	15.0	"	Basal.
21.07	20.60	38.14	38.20	+0.06	15.1	"	11.30 a.m., succinic acid as above.
41.21	39.48	38.36	38.30	−0.06	15.0	"	Basal.
39.83	38.03	38.08	37.91	−0.17	15.1	"	11.45 a.m., 12 gm. acetamide in 200 cc. water.
40.82	36.98	38.26	38.19	−0.07	15.0	"	Basal.

values.

THE SULFUR REQUIREMENT OF THE RED CLOVER PLANT.*

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PLATE 1.

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Several years ago, Hart and Peterson[1] called attention to the apparent deficiency of sulfur in certain types of soil, as related to the demands made upon this element by some species of agricultural plants. More recently, Hart and the writer[2] have reported increased production of dry matter by plants of various species which had received calcium and sodium sulfates in soil cultures conducted in a greenhouse. This effect was particularly striking in the case of common red clover (*Trifolium pratense*), which gave a greater response to calcium sulfate than to sodium sulfate. Other investigators, notably Shedd,[3] have obtained similar beneficial effects from sulfates with soil cultures. Pitz[4] devoted special attention to the effects of calcium sulfate upon the growth of red clover. He found that the development of red clover bacteria, as well as of the young host plant, was stimulated by this source of sulfur.

The present paper deals with the response of the common red clover plant to different forms and planes of sulfur supply, under conditions better controlled than the ordinary soil culture. As nutrient medium, use has been made of the well known nutrient

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., and Peterson, W. H., *J. Am. Chem. Soc.*, 1911, xxxiii, 549.

[2] Hart, E. B., and Tottingham, W. E., *J. Agric. Research*, 1916, v, 233.

[3] Shedd, O. M., *Kentucky Agric. Exp. Station, Bull. 174*, 1914, 595.

[4] Pitz, W., *J. Agric. Research*, 1916, v, 771.

solution of Knop,[5] with a total salt concentration of 0.2 per cent. by volume. It is quite possible that this solution may be improved for the growth of clover by readjusting its salt content, as Shive and also the writer have found with wheat;[6] but, until such readjustment should be tested, it seemed to be as suitable for the present purpose as any other nutrient solution.

The methods followed with the water cultures were those previously employed by the writer,[6] except that the seeds were germinated in pure sand, instead of over water, until the seedlings were large enough for fixing in the culture vessels. About 10 days were required for this stage of development. In order to avoid the high hydrogen ion content which would result from the use of KH_2PO_4 as the only source of phosphorus, this salt and K_2HPO_4 were added to the solution in equimolecular proportions, each in one-half the usual molecular concentration of the KH_2PO_4. Where $CaSO_4$ and $CaCO_3$ were employed they were added in the solid form when the solution was transferred to the culture vessels. Each culture vessel (1 pint jar of the Mason pattern) had a capacity of about 425 cc. and supported nine seedlings at the beginning of each culture series. The nutrient solutions were renewed after intervals of 3 days.

The plan of treatment of the solutions involved both the partial and complete replacement of $MgSO_4$ with $Mg(NO_3)_2$. To the sulfur-free solution produced in the latter case, sulfur was also restored in the form of either Na_2SO_4 or $CaSO_4$, each being made molecularly equivalent to the $MgSO_4$ of the unmodified Knop solution. Still other portions of the sulfur-free solution were modified by adding either $NaNO_3$ or $CaCO_3$, in twice molecular and molecular proportions respectively, as compared with the usual amount of $MgSO_4$. These last modified solutions served as controls for determining the effects of the sodium and extra calcium added elsewhere in the form of sulfates.

The method followed with the sand cultures was that of irrigation with a renewed nutrient solution, as previously employed by McCall.[7] Glazed, stoneware jars, about 16.0 cm. in diameter

[5] Knop, W., *Landw. Versuchssta.*, 1865, vii, 93.

[6] Tottingham, W. E., *Physiol. Researches*, 1913–17, i, 133. Shive, J. W., *ibid.*, 327.

[7] McCall, A. G., *Soil Science*, 1916, ii, 207.

and 11.0 cm. deep (2 quart jars), which conveniently held 3.5 kilos of fine sand, were employed as culture vessels. The inner surface of these jars was coated with paraffin, to prevent contamination of the nutrient solution by solutes from the stoneware. The sand employed consisted of spheroidal particles, most of which passed through a sieve with 70 meshes to the inch but were retained by one of 80 meshes. It was the product designated "banding sand" by the Ottawa Silica Company of Illinois. Although containing only traces of impurities, it was washed thoroughly with water before use. For the purposes of maintaining alkalinity and supplying iron, 3.0 gm. of $CaCO_3$ and 1.0 gm. of Fe_2O_3 were mixed with the portion of sand for each culture. Phosphorus was added to the nutrient solution in the form of K_2HPO_4. The application of nutrient solution was gradually increased from 25 to 100 cc. per culture at each renewal, and the period between renewals was gradually reduced from 4 to 2 days. As measured by Hilgard's method,[8] the water-holding capacity of the sand here employed was 23.4 per cent, by weight. By daily weighings, in connection with the use of a suction pump, the plane of total water content of all of the cultures was made uniform and was increased gradually from 20 to 22 per cent, by weight, of the dry sand. It was possible to measure approximately the loss of water through the plants, as the seedlings and irrigating funnel were sealed about with a mixture of vaseline and paraffin, but the data obtained showed no significant variations.

As none of the plants produced flowers in any of the culture series, it was difficult to select a stage of growth approximating maturity. Each series was harvested, however, when pronounced death of the basal leaves seemed to indicate a decided decline of growth with all of the cultures. After determining the average length of the three or four longest roots, and washing the latter parts, the plants were severed at the crown of the roots. The tops and roots were then dried at about 100°C., for weighing. Except in the case of the sand cultures, the finely ground tops of the duplicate cultures were mixed for determining the average nitrogen content. In some cases, determinations of nitrogen in

[8] Hilgard, E. W., *Soils*, New York, 1906, 208–209.

the form of nitrates were also made. Total nitrogen was determined by the Gunning method[9] and nitrate nitrogen was determined by precipitation with nitron.[10] It seemed possible that the data derived from these analyses would indicate disturbances of protein synthesis, which might follow deficiencies in the sulfur supply of these clover plants.

Series 1 was conducted with water cultures. It covered a much longer period of growth than the succeeding series. After 37 days of growth the cultures were transferred to jars of a capacity of about 850 cc. (1 quart jars of the Mason pattern). The widely varying number of plants which survived in the different cultures tends to make the results of questionable value. However, the latter are unchanged in general trend if considered on the basis of unit plant per culture; and they are confirmed, in some respects, by the succeeding and more uniform series. The results appear in Table I.

Series 2, also conducted with water cultures, was disturbed by attacks from thrips. This may account partly for its comparatively early apparent maturing. Its more restricted growth period may, in turn, account for its less marked differences in yields, as compared with the other series. The results of this series appear in Table II.

Series 3, conducted with sand cultures, reached apparent maturity comparatively quickly. The seedlings were 10 days older than usual when transferred to the cultures, having been nourished for this period by irrigation with the sulfur-free modification of Knop's solution. Only six plants were started in each culture. The entire series was conducted upon a rotating table, after the manner of Shive[11], to render uniform the effects of aerial environment with all of the cultures. The results appear in Table III.

A comparative survey of the results of these three series of cultures shows that 0.9 of the usual amount of $MgSO_4$ in Knop's solution could be displaced, under the conditions of the water

[9] Official and provisional methods of analysis, *Assn. Official Agric. Chemists, U. S. Dept. Agric., Bureau of Chem., Bull. 107* (revised), 1907.

[10] Treadwell, F. P., Analytical chemistry, New York, 4th edition, 1915, ii, 451–453.

[11] Shive, *Physiol. Researches*, 1913–17, i, 344–345.

TABLE I.

Yield and Nitrogen Content of Red Clover (Trifolium pratense) Grown 122 Days (Mar. 13—July 13, 1915) in Knop's Solution and Modifications Thereof.

Culture No.	Description of nutrient medium.	No. of plants matured.	Yield of dry tops.		Yield of dry roots.		Maximal length of roots.		Total nitrogen of tops.		
			Actual.	Relative.	Actual.	Relative.	Actual.	Relative.	Actual.		Relative.
			gm.	*per cent**	*gm.*	*per cent**	*mm.*	*per cent**	*per cent**	*gm.**	*per cent**
1	Unmodified Knop solution.	3	3.252	—	0.924	—	119	—	—	—	—
2		3	3.585	100	1.008	100	103	100	3.47	0.119	100
3	0.9 $MgSO_4$ of Knop solution replaced by	6	3.414	—	0.942	—	137	—	—	—	—
4	$Mg(NO_3)_2$.	2	3.384	99	0.730	87	175	141	3.63	0.123	103
5	Total $MgSO_4$ of Knop solution replaced by	6	2.220	—	1.248	—	250	—	—	—	—
6	$Mg(NO_3)_2$.	8	1.952	61	1.304	132	225	214	4.46	0.093	78
7	Same as 5 and 6 + Na_2SO_4 = $MgSO_4$ of Knop	4	3.388	—	1.044	—	131	—	—	—	—
8	solution.	3	3.075	95	1.113	112	119	113	3.65	0.118	99
9	Same as 5 and 6 + $NaNO_3$ = $MgSO_4$ of Knop	6	2.340	—	1.116	—	162	—	—	—	—
10	solution.	6	2.022	64	1.038	111	225	175	3.76	0.082	69
11	Same as 5 and 6 + $CaSO_4$ = $MgSO_4$ of Knop	4	4.392	—	0.876	—	144	—	—	—	—
12	solution.	6	4.386	128	1.194	107	137	127	3.84	0.169	142
13	Same as 5 and 6 + $CaCO_3$ = $MgSO_4$ of Knop	8	2.072	—	1.208	—	175	—	—	—	—
14	solution.	6	2.004	60	1.266	128	287	208	4.69	0.096	81
15	Same as 5 and 6 + $CaSO_4$ = 3 × $MgSO_4$ of	4	4.352	—	1.200	—	100	—	—	—	—
16	Knop solution.	2	3.708	118	0.904	109	175	124	3.63	0.146	123
17	Same as 5 and 6 + $CaCO_3$ = 3 × $MgSO_4$ of	6	2.454	—	1.434	—	225	—	—	—	—
18	Knop solution.	8	2.496	72	1.520	153	200	192	3.47	0.086	72

* Average value of duplicate cultures.

TABLE II.

Yield and Nitrogen Content of Red Clover Grown 64 Days (Apr. 13—June 16, 1916) in Knop's Solution and Modifications Thereof.

Culture No.	Description of nutrient medium.	No. of plants matured.	Yield of dry tops.		Yield of dry roots.		Maximal length of roots.		Total nitrogen of tops.		
			Actual.	Relative.	Actual.	Relative.	Actual.	Relative.	Actual.		Relative.
			gm.	*per cent**	*gm.*	*per cent**	*mm.*	*per cent**	*per cent**	*gm.**	*per cent**
1	Unmodified Knop solution.	9	1.354	—	0.512	—	137	—	—	—	—
2		9	1.367	100	0.431	100	144	100	3.03	0.041	100
3	0.9 of $MgSO_4$ of Knop solution replaced by $Mg(NO_3)_2$.	9	1.388	—	0.429	—	125	—	—	—	—
4		8	1.289†	98	0.471	95	106	82	3.40	0.046	112
5	Total $MgSO_4$ of Knop solution replaced by $Mg(NO_3)_2$.	9	0.955	—	0.476	—	125	—	—	—	—
6		9	0.861	67	0.470	100	135	92	4.03	0.037	90
7	Same as 5 and 6 + Na_2SO_4 = $MgSO_4$ of Knop solution.	9	1.544	—	0.535	—	150	—	—	—	—
8		8	1.631†	117	0.572	117	150	106	3.20	0.051	124
9	Same as 5 and 6 + $NaNO_3$ = $MgSO_4$ of Knop solution.	8	1.158†	—	0.509	—	160	—	—	—	—
10		9	0.950	77	0.533	108	137	106	3.91	0.041	100
11	Same as 5 and 6 + $CaSO_4$ = $MgSO_4$ of Knop solution.	9	1.271	—	0.517	—	100	—	—	—	—
12		9	1.391	98	0.559	114	128	81	3.24	0.043	105
13	Same as 5 and 6 + $CaCO_3$ = $MgSO_4$ of Knop solution.	9	0.888	—	0.499	—	131	—	—	—	—
14		9	1.046	71	0.521	108	137	95	3.90	0.038	93

* Average value of duplicate cultures.

† Converted to equivalent for nine plants.

TABLE III.

Yield and Nitrogen Content of Red Clover Grown 54 Days (Jan. 7—Mar. 2, 1918) in Sand Irrigated with Knop's Solution and Modifications Thereof, with $CaCO_3$ Added.

Culture No.	Description of nutrient medium.	No of plants matured.	Yield of dry tops.		Yield of dry roots.		Maximal length of roots.		Total nitrogen of tops.		
			Actual.	Relative.	Actual.	Relative.	Actual.	Relative.	Actual.		Relative.
			gm.	*per cent**	*gm.†*	*per cent**	*mm.*	*per cent**	*per cent**	*gm.**	*per cent**
1	Unmodified Knop solution.	6	3.66	—	1.80	—	169	—	4.24	—	—
2		6	3.66	100	1.96	100	138	100	4.25	0.156	100
3	0.99 of $MgSO_4$ of Knop solution replaced by	6	2.88	—	2.08	—	163	—	4.11	—	—
4	$Mg(NO_3)_2$.	6	3.18	83	1.94	107	125	94	3.45	0.114	73
5	Total $MgSO_4$ of Knop solution replaced by	6	1.50	—	0.48	—	150	—	3.43	—	—
6	$Mg(NO_3)_2$.	6	2.22	51	1.12	43	200	114	4.31	0.074	47
7	Same as 5 and 6 + $CaSO_4$ = $MgSO_4$ of Knop	6	5.04	—	2.76	—	169	—	4.04	—	—
8	solution.	6	4.92	136	3.08	155	169	110	4.00	0.201	129

* Average value of duplicate cultures.

† Air-dried; containing sand.

cultures, without seriously disturbing the growth of the red clover plant. In the sand cultures, displacement of 0.99 of the usual amount of $MgSO_4$ led to a considerably decreased production of dry matter of the stems and leaves. Displacement of the total $MgSO_4$ led to decreases of 33 to 49 per cent in the production of dry tops in the various culture series. Addition of sulfur to the modified sulfur-free Knop's solution, in the forms of Na_2SO_4 and $CaSO_4$, resulted in increased growth of the tops of the clover plant. In the majority of these cases the growth was decidedly superior to that with the unmodified Knop's solution. The results of the control cultures with $NaNO_3$ and $CaCO_3$ show that this effect is not attributable to the cations of these added sulfates, when supplied in certain other salts.

As regards the roots, the results are extremely variable. With the water cultures of Series 1 the effect of the various sulfates was to reduce the length of roots, as compared with the sulfur-free modification of Knop's solution, but this was not true of the other series of cultures.

The amount of nitrogen absorbed by these cultures fluctuated more or less parallel to the yields of dry tops, both of these values decreasing with the decrease of sulfur supply. It would seem logical to conclude that a deficiency of sulfur here operated to decrease the synthesis of protein and restrict the elaboration of plant tissue, with a concomitant decrease in the assimilation of nitrogen from the culture medium. That the percentage of assimilation of the absorbed nitrogen was not greatly different in the various cultures of each series here dealt with, is shown by the following percentages of the total nitrogen in the form of nitrate, as determined by analysis: Series 1, Cultures 1 and 2, 7.2 per cent; Cultures 3 and 4, 11.0 per cent. Series 3, Cultures 1 and 2, 7.5 per cent; Cultures 5 and 6, 6.2 per cent; Cultures 7 and 8, 7.5 per cent.

On the whole, the results of these cultures show a superiority of Na_2SO_4 and $CaSO_4$ over $MgSO_4$, as sources of sulfur for the red clover plant in water and sand cultures. This might be expected to follow the known toxic properties of magnesium salts. In harmony with the results previously reported from soil cultures[2] $CaSO_4$ has proved an especially efficient source of sulfur.

In view of the fact that all of the nutrient media which here supplied sulfur were liberally supplied with calcium, it appears that $CaSO_4$ had peculiar efficiency as a molecular complex.

SUMMARY.

The data presented here show that, for these experimental conditions, between 0.1 and 0.01 of the usual amount of $MgSO_4$ of Knop's solution was as efficient as the usual amount for the growth of red clover, when the balance of the $MgSO_4$ was replaced by $Mg(NO_3)_2$. The yield of dry tops of this plant was only one-half to two-thirds as great with $MgSO_4$ wholly replaced by $Mg(NO_3)_2$ as with the unmodified Knop's solution. Addition of Na_2SO_4 and $CaSO_4$ to the sulfur-free modification of Knop's solution, in amounts equivalent to the sulfur of the unmodified solution, produced yields of dry tops superior to those of the latter solution. In this respect, $CaSO_4$ was very efficient. From the nature of the various nutrient media employed, it appears that the sulfur of this salt functioned in the molecular combination in which it was supplied. The more or less parallel fluctuations of plane of sulfur supply, weight of nitrogen assimilated, and yield of dry tops, of these clover plants, indicate that a deficiency of sulfur supply restricted growth by limiting the synthesis of protein.

EXPLANATION OF PLATE 1.

FIG. 1. Series 1. Water cultures of *Trifolium pratense* after 99 days of growth. Plants 1 and 2, unmodified Knop's solution. Nos. 5 and 6, total $MgSO_4$ of Knop's solution replaced by $Mg(NO_3)_2$. Plants 7 and 8, same as Nos. 5 and 6, plus $Na_2SO_4 \Rightarrow MgSO_4$ of Knop's solution. Plants 11 and 12, same as Nos. 5 and 6, plus $CaSO_4 \Rightarrow MgSO_4$ of Knop's solution. Plants 13 and 14, same as Nos. 5 and 6, plus $CaCO_3 \Rightarrow MgSO_4$ of Knop's solution.

FIG. 2. Series 3. Sand cultures of *Trifolium pratense* after 53 days of growth. Plants 1 and 2, unmodified Knop's solution. Nos. 3 and 4, 0.99 of $MgSO_4$ of Knop's solution replaced by $Mg(NO_3)_2$. Plants 5 and 6, total $MgSO_4$ of Knop's solution replaced by $Mg(NO_3)_2$. Plants 7 and 8, same as Nos. 5 and 6, plus $CaSO_4 \Rightarrow MgSO_4$ of Knop's solution.

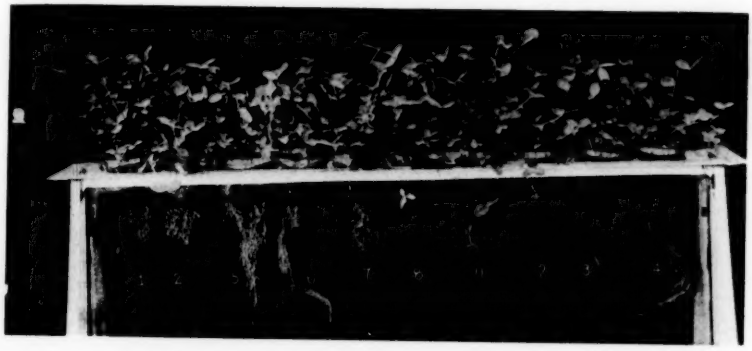

FIG. 1.

FIG. 2.

(Tottingham: Red clover.

STUDIES OF EXPERIMENTAL SCURVY.

III. THE INFLUENCE OF MEAT AND VARIOUS SALTS UPON THE DEVELOPMENT OF SCURVY.*

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Gerstenberger[1] in a recent paper upon scurvy attributes the cause of the disease entirely to a deranged calcium metabolism. He states: "But it has seemed to me, in view of the known data regarding the pathology, chemistry and symptomatology of scurvy on the one hand, and regarding the important function played by calcium in bone growth, nerve conduction, vessel sealing, and cell permeability on the other, that all of these different system symptoms might be explained on the common basis of an interference with one or more of the normal functions of calcium and its physiological anion in the osseous system, vascular system, nervous system, muscular system and probably other systems." That calcium can play an important part in scurvy prevention is shown by the evidence presented in this paper, but that its metabolism alone is involved is questionable in the light of previous work from this laboratory.[2,3] Gerstenberger questions whether the guinea pigs that McCollum and I reported upon had only scurvy, and is of the opinion that they may also have had rickets. Our guinea pigs had the symptoms that in the literature are described as being characteristic of scurvy.

It is possible that the guinea pigs, or at least some of them may have had both scurvy and rickets. In my work I have found that guinea pigs kept on the same ration do not always show the same symptoms, but that they vary to a certain degree. This

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Gerstenberger, H. J., *Am. J. Med. Sc.*, 1918, clv, 253.

[2] McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.

[3] Pitz, W., *J. Biol. Chem.*, 1918, xxxiii, 471.

has brought doubt in my mind as to whether or not what is commonly called scurvy in guinea pigs may not in reality be a complication of symptoms that overlap both scurvy and rickets, since both have a number of features in common.

Baumann and Howard[4] in a study of the mineral metabolism of normal and scorbutic guinea pigs found that all elements except magnesium and chlorine showed a negative balance during the scorbutic period. Bahrdt and Edelstein[5] in a chemical analysis of bones from severe cases of scurvy found a distinct deficiency of calcium and phosphorus in the bones and muscles. Lust and Kloeman[6] on the other hand, found that in a severe case of scurvy in an infant there was a retention of calcium, phosphorus, and chlorine, and especially of calcium during the active stage of the disease. During the convalescent stage of the case they found a negative mineral balance produced by the excessive excretion of salts and finally that in the period of healing the mineral balance was positive, except in the case of calcium, which was still negative. The differences in the findings of the above investigators is explained by Gerstenberger who showed that there is an increase in the calcification at Fränkel's line, indicating that scurvy is not a calcium deficiency disease; that while there is an excess of calcium deposited at that part of the bone at which endochondral growth takes place under normal conditions, there is nevertheless in all probability not so much calcium in the bone as a whole in advanced stages of scurvy as there would be in the normal bone; because, first, the bone has ceased to grow entirely or at any rate does not grow so rapidly as under normal conditions, and second, the normal process of resorption is going on, producing a rarefication and brittleness with consequent loss of material.

Hess and Fish[7] have shown that the blood of infants suffering with scurvy shows a slight diminution in clotting power but this defect did not seem to be the result of an insufficiency of calcium. There was also an increased permeability of the vessel walls. Calcium is known to have a distinct function in controlling the

[4] Baumann, L., and Howard, C. P., *Am. J. Med. Sc.*, 1917, cliii, 650.
[5] Bahrdt, H., and Edelstein, F., *Z. Kinderh.*, 1913, ix, 415.
[6] Lust, F., and Kloeman, L., *Jahrb. Kinderh.*, 1912, lxxv, 663.
[7] Hess, A. F., and Fish, M., *Am. J. Dis. Child.*, 1914, viii, 385.

permeability of animal membranes,[8–12] and any interference with this function of calcium would increase the permeability of such membranes. Evidence of an increased permeability of the intestinal walls is shown by the fact that the blood serum of some guinea pigs suffering with scurvy gives positive precipitin tests to the proteins contained in the ration. This will be discussed in detail below.

A number of investigators have attempted to prevent the development of scurvy by the administration of phosphorated cod liver oil and tricalcium phosphate. Holst and Fröhlich[13] fed guinea pigs calcium in different forms without getting any beneficial results, and Gerstenberger[1] fed cod liver oil containing tricalcium phosphate, but without result. However, I have been able to protect guinea pigs against scurvy for 18 weeks by the ingestion of meat and tricalcium phosphate in a ration of rolled oats and milk. When tricalcium phosphate was fed in the above ration without the meat there was some beneficial effect noticed but it was not nearly so pronounced as when meat was introduced.

Hart, Miller, and McCollum[14] found that pigs fed a ration of corn, wheat middlings, oats, and oil meal, with a small quantity of roots and alfalfa hay included daily, grew at a slow rate for 4 to 5 months, after which growth ceased and the animals passed into a poor condition accompanied by lack of muscular coordination, emaciation, and labored breathing. The animals were unable to stand. When 1 per cent of commercial meat scraps was added to the above ration the animals grew at a normal rate, were vigorous, and in splendid condition, but the young were carried 1 or 2 weeks over time and were born dead. When 5 per cent of meat scraps was added the animals grew at a normal rate, were in excellent condition, and gave birth to fairly good litters. Some of the young, however, were weak and died. The

[8] Chiari, R., *Arch. exp. Path. u. Pharmakol.*, 1910, lxiii, 434.

[9] Chiari, R., and Januschke, H., *Arch. exp. Path. u. Pharmakol.*, 1911, lxv, 120.

[10] Overton, E., *Arch. ges. Physiol.*, 1904, cv, 176.

[11] Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 139.

[12] Loeb, *J. Biol. Chem.*, 1915, xxiii, 423.

[13] Holst, A., and Frölich, T., *Z. Hyg. u. Infektionskrankh.*, 1912, lxxii, 1.

[14] Hart, E. B., Miller, W. S., and McCollum, E. V., *J. Biol. Chem.*, 1916, xxv, 239.

remarkable influence of the meat scraps is attributed by Professor Hart to an improvement of the proteins and to an appreciable increase in the calcium and phosphorus content of the ration through the introduction of bone material in the meat scraps. It was because of the splendid supplementary action of the meat scraps as shown by the work quoted above that the author investigated the effect of meat and tricalcium phosphate upon the development of scurvy.

The meat used in these experiments was lean beef that was obtained at a local butcher shop. The fat and connective tissue were cut away and the lean beef was put through the meat chopper. It was then dried in shallow granite pans over steam coils. After the meat was dry it was again ground in the meat chopper and stored in a tightly stoppered bottle. The rolled oats were ground up fine and the meat and salts mixed into it, so that the animals could not sort out any of the ingredients of the ration. All the animals in these experiments received fresh milk which was fed *ad libitum*.

Chart 1, Lot 80, shows the effect of the ingestion of 5 per cent of dried meat in the rolled oats and milk ration. All the animals on this ration developed scurvy during the 4th and 5th weeks except No. 5. This guinea pig died at the end of the 3rd week and showed lesions of scurvy. The other animals, although they developed scurvy during the 4th and 5th weeks, lived for a considerable time but did not gain in weight. Animals 7 and 8 were killed at the end of the 17th week. The meat apparently did not delay the onset of the disease to an appreciable degree since animals fed rolled oats and milk will develop scurvy during the 3rd and 4th weeks. *The meat did, however, greatly prolong the life* of these animals, since guinea pigs fed on a diet of rolled oats and milk seldom live more than through the 5th week. The meat may have supplemented the proteins of the oat and milk ration, or it may have acted as a stimulant to the appetites of the animals. One can hardly see how the meat served as an antiscorbutic in a capacity similar to phenolphthalein or mineral oil, as pointed out in a previous paper. One can, however, surmise how it may have supplemented a partially incomplete and already deficient intake of protein and thereby increased the constitutional resistance of the animal, and how it may also have stimulated appe-

tite with its resultant flow of digestive juices and concurrent proper alimentation. The element of increased resistance is not to be overlooked, especially when it is noted that while the time of onset of the disease was not delayed the period of life was prolonged considerably. It is certain that the protein intake on the basal ration of rolled oats and milk was far from satisfactory. In an experimental group of animals kept on sand to avoid consumption of litter a careful record of the proportionate amounts of food consumed indicated a protein intake equivalent to 10.2 per cent of the solids in the ration. Of this amount of protein 7.7 per cent was oat proteins and 2.5 per cent milk proteins. A comparison of the records of this group of animals with the records of animals given in Charts 3, 13, 14, and 15 demonstrates beyond a doubt that the meat acted primarily in improving and raising the level of the proteins of the ration. Since meat did not delay the onset of the symptoms of scurvy but did prolong the life of the animals it is highly probable that the inadequate nature or insufficient amount of proteins in the oat and milk ration may have been as much the cause of the death of the animals suffering from scurvy as was the disease. The food consumption of animals suffering with scurvy is very small during the active stages of the disease and the protein consumption is low at that time.

The guinea pigs in Chart 2, Lot 90, received a ration of rolled oats, meat, tricalcium phosphate, and milk during Period I. Three of these animals remained in excellent condition for 18 weeks and grew at a slow rate during that time. The animals were active, their food consumption was high, and they appeared normal in all respects. Animal 4 died at the end of the 4th week, but postmortem examination failed to show any of the characteristic lesions of scurvy. The tract of the animal was empty and it probably died of starvation. Animal 5, which was started the 13th week, showed slight symptoms of scurvy during its 5th week on the ration. It also had prolapse of the rectum. During the 18th week all animals lost weight and all but No. 1 developed scurvy. No. 2 died during the 19th week. In Period II 1 per cent of sodium chloride was added to the ration. The animals immediately improved in appearance, became more active, regained their appetites, and increased in weight, but the symptoms of scurvy remained. From the 18th to the 20th week the

food consumption of this group of animals had fallen off rapidly, but when NaCl was added to the ration their food consumption increased. 4 weeks after the introduction of NaCl into the ration the guinea pigs again began to lose weight and Animal 5 died 5 weeks later. It showed severe lesions of scurvy. Nos. 1 and 3 were killed at the end of the 30th week. Animal 1 did not show any lesions of scurvy but No. 3 did. The decided improvement of this ration over that of one of oats and milk is due in part to the 5 per cent of meat contained in the ration as shown by Chart 1, Lot 80, but whereas the animals in Lot 80 developed scurvy during the 4th and 5th weeks only two of the five animals in this group (No. 4 died at the end of the 5th week) developed scurvy before the 18th week. Clearly then the delay in the development of the disease was due to the 1 per cent of tricalcium phosphate in the ration. Calcium is known to function in maintaining normal permeability of animal tissues and it is in this capacity that the calcium probably acted. That there is an increase in the permeability of the intestinal and vessel walls of infants and guinea pigs suffering with scurvy is an established fact, and since calcium phosphate delayed the onset of the disease for a considerable time, the supposition seems justified that the calcium acted in maintaining normal permeability of these tissues and thus lessened the absorption of toxic products from the cecum and large intestine.

Calcium phosphate was found to be superior to other calcium salts, except perhaps calcium chloride, in delaying the onset of the symptoms of scurvy (see Charts 7, 8, 9, and 11). Since there is an abnormal accumulation of calcium at Fränkel's line, as shown by previously cited work, the calcium for this abnormal calcification may have been supplied by the calcium phosphate in the ration, because it is principally calcium phosphate that is concerned in bone formation; for this reason other calcium salts would not be as efficient. The case of calcium chloride will be discussed later in this paper.

During the disease the digestive tract is in an abnormal condition, the cecum and large intestine are greatly distended with putrefying feces, and normal peristalsis is interfered with. We may expect at the same time a decreased secretion of gastric juice and as a result a deficiency of HCl. The abnormal retention

of chlorine found to exist in an infant suffering with scurvy as reported by Lust and Kloeman[6] indicates an abnormality in chlorine metabolism. That an actual deficiency of chlorine did exist in the ration fed these guinea pigs is shown by the fact that the ration contained only 0.075 per cent of chlorine. Dr. Babcock[15] showed that when milch cows were deprived of sodium chloride for long periods of time they soon reached a condition of low vitality in which a sudden and complete breakdown occurred. Recovery was rapid if NaCl was supplied. The condition was marked by loss of appetite, a lusterless eye, rough coat, and a very rapid decline in body weight.

The guinea pigs in this group, just previous to the addition of NaCl to the ration, behaved in a manner similar to that of the cows fed by Dr. Babcock; as in the case of the cows, recovery of the guinea pigs was rapid after NaCl was fed. This indicates that the manner in which the NaCl probably acted was in furnishing chlorine for greater HCl production in the gastric juice and in supplying a deficiency of this essential element with a concurrent increase in the constitutional resistance and improvement of the general well being of the guinea pigs.

The animals in Lot 91 (see Chart 3) received 10 per cent of meat in the ration. Animal 3 died during the 8th week and showed lesions of scurvy. The other animals did not show symptoms of scurvy until the 13th week. Animal 4 died at the end of the 16th week and No. 2 died at the end of the 24th week. Animal 1 was killed at the end of the 28th week and showed lesions of scurvy. When 5 per cent of meat was added to the rolled oats and milk ration the onset of the disease was not greatly delayed, but the life of the animals was prolonged. When 10 per cent of meat was added the onset of the disease was greatly delayed and the life of the animals was prolonged. This record is conclusive proof that the meat improved the ration through the introduction of higher levels and improvement of the proteins and by virtue of an increased intake of extractives and secretagogues which resulted in greater stimulation of appetite and its resultant increased flow of digestive juices. By improving the proteins and thus making the ration more nearly adequate for the physiological

[15] Babcock, S. M., *Wisconsin Agric. Exp. Station, 22nd Ann. Rep.*, 1905.

demands, and by stimulating the appetite and secretion of digestive juices, the resistance of the animals was increased and they were able to ward off the disease for a long time.

Chart 4, Lot 96, shows the result of feeding a ration of rolled oats, tricalcium phosphate, and milk. When the ration was not fortified with more protein, the tricalcium phosphate was unable to protect the animals to any marked degree. Animal 4 died at the end of the 2nd week and No. 5 at the end of the 6th week. Both of these animals died of scurvy. Nos. 2 and 3 showed symptoms of scurvy during the 5th week, but after the 6th week they increased in weight and probably would have lived for some time had they not been killed at the end of the 9th week for samples of blood. The tricalcium phosphate did delay the onset of scurvy to a very slight degree and prolonged the life of some of the animals. Apparently it is not so potent in delaying the onset of the disease as are lactose and laxatives,[2] and therefore unless the ration is improved as to its protein the tricalcium phosphate is not so effective as when protein is added to the ration.

In order to determine whether the calcium or the phosphoric acid radical or both were effective in causing the delay in the onset of the symptoms of scurvy other calcium salts and other salts of phosphoric acid were fed with the ration of rolled oats, meat, and milk. Chart 5, Lot 100, shows the effect of the addition of 1 per cent of dipotassium phosphate to the above ration. Animal 1 died at the end of the 3rd week and showed lesions of scurvy. No. 4 showed evidences of having the disease during the 4th week and died at the end of the 6th; No. 5 developed scurvy during the 10th week and died the 24th week; No. 6 never showed any signs of having scurvy. It died at the end of the 20th week, but no lesions of scurvy were observed. Since all the animals developed scurvy during the 4th week, except No. 5, which developed it during the 10th week, and No. 6, which never developed the disease, these animals did little better than those that received only the oats plus 5 per cent of meat. It is clear from this record that the dipotassium phosphate was of little benefit. Animal 6 apparently was an exceptional animal. Occasionally an animal will thrive on oats and milk. These results indicate that it is the calcium and not the phosphoric acid that is of prime importance in protecting the animal against scurvy.

To test further whether or not the calcium is instrumental in protecting the animals against scurvy, disodium phosphate was fed with the rolled oats, meat, and milk diet. Chart 6, Lot 103, gives the data of these experiments. The results in this case were no better than those obtained with the dipotassium phosphate. All the animals except No. 3 developed scurvy during the 4th week. No. 1 died at the end of the 5th week; No. 2 at the end of the 8th week, and No. 4 at the end of the 15th week. Animal 3 grew well and appeared to be normal, but when it was killed at the end of the 19th week a postmortem examination revealed a few lesions of scurvy. This record furnishes added proof that it is not the phosphoric acid, or at any rate not the phosphoric acid alone, that is instrumental in delaying the symptoms of scurvy.

The previous two records indicate that phosphoric acid alone played little or no part in protecting the guinea pigs against scurvy. Calcium alone or a combination of calcium and phosphorus must be necessary for protection against the disease. To analyze this point several other calcium salts were fed to guinea pigs with the rolled oats, meat, and milk ration. One group of animals (see Chart 7, Lot 104), was fed calcium acetate with the above ration. The amounts of calcium fed were the same in all these lots. The record of these animals indicates that although calcium is more effective in protecting the animals against scurvy than phosphoric acid, yet calcium acetate was not nearly so effective as was tricalcium phosphate. Since neither the calcium alone nor phosphoric acid was very effective in protecting these animals, the beneficial effects derived from tricalcium phosphate must have been due to that molecule rather than to any particular radical thereof. All the animals that received the calcium acetate developed scurvy about the 4th week. Animal 1 died at the end of the 4th week, No. 2 at the end of the 16th week, No. 3 at the end of the 12th week, and No. 5 at the end of the 5th week. Animal 4 grew at a rapid rate for 12 weeks and at no time did it show any symptoms of scurvy. It was killed at the end of the 20th week and on postmortem examination showed slight indications of scurvy.

Chart 8, Lot 105, shows the result of feeding a group of guinea pigs the above ration, but containing calcium carbonate in place

of calcium acetate. The results with these animals were much the same as those observed with the animals that received calcium acetate. All the animals developed scurvy during the 5th week and No. 1 died at the end of that week. Animal 3 died at the end of the 11th week. Up to the 10th week the other two animals grew a little, after which they did litttle better than maintain their weight.

The animals that received calcium lactate in place of calcium carbonate or acetate did a little better than did the animals on the previous two rations. Compare Chart 9 with Charts 7 and 8. Animal 1 died during the 3rd week, but failed to show any lesions of scurvy. The animal probably died of starvation. Only one animal, No. 2, showed symptoms of scurvy during the 4th week, but this animal lived for 20 weeks and grew rapidly throughout this period. When the animal was killed at the end of the 20th week it showed severe lesions of scurvy. No. 3 grew rapidly from the 10th week on, and never showed any evidence of having scurvy, nor could any lesions of scurvy be found when it was killed at the end of the 20th week. Animal 4 developed the disease during the 7th week and died at the end of the 9th week. Animals 5 and 6 developed scurvy during the 7th week and No. 6 died at the end of the 7th week, while No. 5 lived until the 16th week.

These results confirm the supposition that the beneficial effects of the tricalcium phosphate are due to the tricalcium phosphate molecule as a whole rather than to either the calcium or the phosphoric acid radical alone. Monobasic calcium phosphate was found to be less effective in delaying the development of scurvy than was tricalcium phosphate.

It will be recalled that the guinea pigs in Lot 90 (see Chart 2) began to lose weight, became inactive, and developed scurvy about the 18th week, and that when NaCl was added to the ration the animals improved within a week and gained in weight, but the symptoms of scurvy persisted. These animals already had severe scurvy, so that it is possible that the NaCl was added too late to be of any great aid to them. In order to determine whether or not guinea pigs would be entirely protected by the ingestion of NaCl in a ration of rolled oats, meat, tricalcium phosphate, and milk when fed to young animals at the beginning

of the feeding trial, a group of young guinea pigs was started on that ration (see Chart 10, Lot 117). All the animals suffered from diarrhea at the beginning of the experiment and two of them died. The other two animals recovered after the 6th week. Two additional animals were started on the ration 9 weeks later. One of these animals, No. 5, lost weight during the first 7 weeks, after which it gained in weight rapidly for a time and then decreased in its rate of growth. At no time, however, did this animal show symptoms of scurvy; No. 6 grew rapidly for 5 weeks and then declined in weight. All the animals in this group lost weight during the last 4 weeks of the feeding trial. The weather was very hot during this time, which was probably responsible for the decline in weight. However, only one animal, No. 4, showed positive symptoms of scurvy, while Animal 1 was questionable.

The records of this group of animals, as well as those of the animals given in Charts 11 and 12, are rather unsatisfactory due to the very hot weather prevailing during the experimental period. The animals were discarded sooner than they should have been because the author was called into military service. The results of the records of Chart 10, however, confirm those of Chart 2; namely, that $Ca_3(PO_4)_2$ and NaCl when fed with a ration of rolled oats, meat, and milk to guinea pigs greatly prolong the life and well being of the animals.

Since chlorine was found to be beneficial when fed with tricalcium phosphate in the oats and meat ration it was deemed advisable to feed calcium chloride with the oats, meat, and milk ration. Chart 11, Lot 118, is the record of these feeding trials.

After a slight decline in weight during the first 3 weeks of the feeding trial all the animals, except No. 4 which died at the end of the 2nd week, grew at a rapid rate. Animal 5 died at the end of the 6th week, but showed no lesions of scurvy. The remaining three animals grew very rapidly until the 10th week, after which time two of them declined in weight, but No. 2 continued to grow at a somewhat slower rate. Animals 1 and 3 showed slight symptoms of scurvy, but No. 2 never showed any signs of having the disease.

A comparison of the curves of these animals with those of the animals that received $Ca_3(PO_4)_2$ and NaCl shows that $Ca_3(PO_4)_2$ and NaCl are superior to $CaCl_2$ in protecting guinea pigs against

scurvy when fed with rolled oats, meat, and milk. However, it must be remembered that the animals of Chart 2 were kept under more favorable conditions than were the animals of Chart 11. The animals of Chart 10, however, and those of Chart 11 were kept under similar conditions and the records of these two groups of animals can therefore be compared. A comparison of the records of these two groups of animals shows clearly that $CaCl_2$ is somewhat superior to $Ca_3(PO_4)_2$ and NaCl in delaying the onset of scurvy in guinea pigs when fed with a ration of rolled oats, meat, and milk. These results demonstrate that it is calcium and chlorine that are mainly instrumental in delaying the onset of scurvy in guinea pigs and that the phosphorus plays a less important rôle in the development of this disease. They also show that calcium and chlorine when supplied in the same molecule as in $CaCl_2$ are more effective in delaying the onset of the disease than when they are supplied in separate molecules as in $Ca_3(PO_4)_2$ and NaCl.

In order to determine whether guinea pigs will do just as well when fed calcium chloride as when fed tricalcium phosphate and NaCl with the oats and milk ration, one lot of animals was fed calcium chloride with rolled oats and milk and another lot was fed tricalcium phosphate and NaCl with rolled oats and milk (see Chart 12, Lots 119 and 120).

A comparison of the two sets of records in Chart 12 shows that the $CaCl_2$ when fed to guinea pigs with the rolled oats and milk ration is far superior to $Ca_3(PO_4)_2$ and NaCl in delaying the onset of scurvy. Those animals that received $Ca_3(PO_4)_2$ and NaCl, Ration 119, did little better than animals kept on rolled oats and milk. Of course had these animals been kept under more favorable circumstances the records would have been better, but since both of the groups in Chart 12 were kept under the same conditions a comparison of the two sets of records is justifiable. All the animals of Ration 119, except No. 4, died before the end of the 5th week. No. 4 lived until the end of the 9th but showed no lesions of scurvy. The animals of Ration 120, that received $CaCl_2$, grew at a slow rate and were in much better condition than were the animals of Ration 119. Animal 1 died at the end of the 9th week, but showed no symptoms of scurvy; No. 3 was killed at the end of the 4th week and showed slight symptoms of scurvy.

Animals 2 and 5 lived for 14 weeks and grew at a slow rate throughout the feeding trial. No. 2 showed slight symptoms of scurvy the 9th week, but No. 5 never showed lesions of the disease. The animals were discarded at the end of the 15th week.

These records clearly demonstrate the superiority of $CaCl_2$ over $Ca_3(PO_4)_2$ and NaCl in delaying the onset of scurvy in guinea pigs and illustrate how much more effective certain ions may be when combined in the same molecule than when present in different molecules.

Since $CaCl_2$ was found to be superior to $Ca_3(PO_4)_2$ and NaCl in delaying the onset of scurvy in guinea pigs, calcium and chlorine must be of greater importance than phosphorus in the etiology of scurvy, and in this disease we must, therefore, have present a deficiency of chlorine as well as of calcium, or at least a decided derangement of chlorine metabolism. Such a deficiency or derangement of chlorine metabolism must result in a deficient production of HCl and a decrease in the digestive power of the gastric juice with a resultant improper functioning of the digestive tract.

To demonstrate further that the meat ingested with the ration of rolled oats and milk acted primarily in improving the proteins of the ration various other proteins were fed with rolled oats, milk, and tricalcium phosphate. Chart 13, Lot 101, is the record of a group of guinea pigs fed the above ration, plus 5 per cent of casein. That the casein did supplement the protein of the ration is evidenced by the results of this feeding trial (compare with Chart 4). Animal 5 developed scurvy the 3rd week and died at the end of that week; No. 4 developed the disease during the 9th week and died the 11th. None of the other animals showed any evidence of having scurvy. No. 2 died at the end of the 8th week, but no lesions of scurvy could be found, and No. 6 died at the end of the 7th week, but showed no evidences of having had the disease. Animals 1 and 3 grew at a rapid rate throughout the time of the experiment and appeared to be normal in all respects. They were killed at the end of the 24th week and showed no lesions of scurvy. The record of these animals is not so good as is that of animals fed meat in place of casein (see Lot 90, Chart 2). The meat therefore must have acted in a capacity other than simply in improving and increasing the protein content of the

ration to account for its superiority over the casein. What this was cannot be definitely pointed to at this time, but as stated before it probably stimulated the appetites of the animals and also stimulated secretions in the tract. Pawlow[10] showed that certain extractives found in meat stimulated a copious flow of gastric juice.

Chart 14, Lot 113, shows the result of feeding wheat gluten in place of casein in the above ration. As is to be expected the animals that received the wheat gluten did not do so well as those that received casein. Three of the animals developed scurvy the 5th week, while two of the animals did not develop the disease at all. The wheat gluten proved effective not in supplementing the other proteins of the ration, but simply in increasing the total protein content of the ration.

Liebig's extract was fed with the rolled oats, tricalcium phosphate, and milk ration to determine whether or not the meat had acted through its extractives as well as through the protein. Chart 15, Lot 114, gives the results of these experiments and shows that some beneficial effects were obtained by feeding the meat extract. The results were not nearly so good as those obtained with the meat or casein (see Charts 2 and 14). The animals all developed scurvy during the 4th and 5th weeks and all but one did very poorly throughout the feeding trial, but the life of these animals was prolonged. The protein contained in the meat extract was in part responsible for the beneficial effects noted, but the protein content of the meat extract was probably too low to account wholly for so great an effect as was noted.

Precipitin Tests.

In investigations on the effect of various rations on the growth of swine, Professor Hart has observed that this class of animals will pass into pathological conditions when confined to corn and milk. These animals suffer from severe constipation, dragging of the hind quarters, and swollen joints. He further showed[16] that the blood of these animals gave positive precipitin tests to corn and milk proteins, indicating an invasion of the blood stream through the intestinal walls of unhydrolyzed proteins of the ration.

[16] Unpublished data from this laboratory.

Since many of the symptoms that are shown by swine, kept on diets restricted to grains and small amounts of roughage or milk, are similar to some of the symptoms shown by guinea pigs suffering with scurvy, namely constipation, dragging of the hind legs, and swelling of the joints, precipitin tests were made upon the serums of scurvy guinea pigs. Fresh skimmed milk and oat protein were used for these tests and in some cases other proteins. To prepare the oat protein, finely ground rolled oats were stirred into hot, distilled water and allowed to cool. The mixture was then centrifuged and filtered through a Berkefeld filter. A clear, slightly yellowish solution was thus obtained. The milk and oat protein solutions were sterilized in an autoclave for 20 minutes at 15 pounds pressure. For the test with milk proteins 1 cc. of sterile milk was diluted with 50 cc. of sterile distilled water. All the tests were purely qualitative in nature, and no titrations were made to determine the amounts of precipitin contained in the blood serum. 1 cc. of the serum was diluted with 10 cc. of sterile, distilled water. The thoracic cavity of the animals was opened and the blood from the heart allowed to flow into a sterile Petri dish. After the blood had coagulated the clear serum was drawn off with a sterile pipette. For controls, the blood serum of normal guinea pigs kept on a diet of rolled oats and fresh grass was used.

In no case was a positive test observed with the serum of normal guinea pigs. The results of these tests are given in Table I. The corn and wheat proteins were prepared in the same manner as were the oat proteins. The egg protein was prepared by dissolving dried egg albumin in distilled water. All the protein solutions were sterilized.

A review of the data given in Table I shows that none of the animals that did not have scurvy gave positive precipitin tests with the proteins investigated, and that, while some of the animals that did have scurvy gave positive precipitin tests, others did not. All of the animals that had severe scurvy, except one, gave positive tests, although some gave strong, while others gave rather weak tests; in some cases the tests were so weak as to leave doubt as to whether the reaction was really positive. It is clear from this table that the stage of the disease apparently has no influence upon the response to the precipitin test. All that can be concluded thus far is that while normal guinea pigs do not

give a positive precipitin test to oat and milk proteins some scurvy animals do, but that the test is no indication of the severity of the disease. The precipitin test for oat and milk proteins was not specific, but also was given by corn and wheat protein. It has been found by several investigators that precipitins are

TABLE I.

Results of the Application of Precipitin Tests to the Serum of Normal and Scurvy Guinea Pigs.

	Ration.	Stage of disease.	Protein.				
			Oat.	Corn.	Wheat.	Milk.	Egg.
1	Oats and grass	Normal.	0	−	−	0	−
2	" " "	"	0	−	−	0	−
3	" " "	"	0	−	−	0	−
4	" " "	"	0	−	−	0	−
5	" " milk	Severe scurvy.	++	−	−	++	−
6	" " "	" "	++	−	−	++	−
7	" milk, and $CaCl_2$	Slight "	+	−	−	+	−
8	" and milk	Severe "	+	−	−	+	−
9	" meat, milk, and $CaCO_3$	" "	?	−	−	?	−
10	" and milk	Slight "	0	−	−	0	−
11	" meat, and milk	" "	+	+	+	+	0
12	" " " "	" "	+	+	+	+	0
13	" Liebig's extract, $Ca_3(PO_4)_2$, milk	Severe "	0	−	−	0	0
14	" heated meat, $Ca_3(PO_4)_2$, milk	" "	+	+	+	+	0
15	" wheat gluten, $Ca_3(PO_4)_2$, milk	" "	?	−	−	?	−
16	" casein, $Ca_3(PO_4)_2$, milk	No "	0	−	−	0	−
17	" meat, Na_2HPO_4, milk	Slight "	+	−	−	+	−

0 = no tests given
++ = strongly positive.
+ = positive.
? = so slight as to be questionable.
− = no tests made.

not always specific.[17] The data here reported are still incomplete and are given merely as a preliminary report. The problem is being investigated further in this laboratory.

[17] Longcope, W. T., *Harvey Lectures*, 1915–16, xi, 271.

The presence of certain foreign bodies that give positive precipitin tests to oat, corn, wheat, and milk proteins in the blood stream of some guinea pigs suffering with scurvy gives rise to the question as to whether their presence in the blood is due to an increased permeability of the tissues, or to lesions in the intestinal walls. From the accumulation of evidence that in scurvy there is an increase in the permeability of the tissues, and in view of the fact that an ingestion of calcium phosphate or calcium chloride into the diet delayed the development of the disease, it is highly probable that the presence of foreign proteins in the blood stream is probably due to the increased permeability of the tissues. This does not exclude the possibility of lesions in the intestines playing a part in allowing protein molecules to pass into the blood stream. In order to determine whether lesions do exist in the intestinal walls of guinea pigs suffering with scurvy an examination of the intestinal walls of scurvy animals is being made in this laboratory.

SUMMARY.

The evidence given in the three papers upon scurvy from this laboratory shows that while the physical character of the diet is of great importance in preventing scurvy other factors enter into the causes of the disease.

In this paper it is shown that an improvement of the protein of the diet will protect guinea pigs from scurvy for a number of weeks and will greatly prolong the life of the animals, even though the physical character of the diet has not been improved. But while laxatives[2] and lactose[3] will prevent the development of scurvy in guinea pigs for 20 weeks, improvement in the protein of the diet, even when 10 per cent of meat is added, will not protect them for more than 13 weeks. When tricalcium phosphate was added with the meat the animals were protected for 18 weeks. Sodium chloride when added to the milk, rolled oats, meat, tricalcium phosphate ration, also effected some further protection, demonstrating that low chlorine is one among the many factors that may cause delay in the development of scurvy. This latter point is further emphasized by the remarkable protection afforded guinea pigs against scurvy by the ingestion of $CaCl_2$ in the ration and shows that the calcium and chlorine ions are of greater importance in the development of this disease than is phosphorus.

The physical character of the diet and the character of the flora of the digestive tract are clearly of prime importance in the production of this disease, but other factors, such as those which make the diet more nearly chemically complete, which stimulate appetite and increase the flow of digestive juices and increase the resistance of the animals, which decrease the permeability of the intestinal wall, and which aid in correcting a deranged chlorine metabolism, are of great importance and will protect the animals from scurvy for a considerable time. *These experiments point to the little emphasized rôle of calcium salts in nutrition; namely, that of controlling the permeability of various animal tissues and thereby affording protection against invading agents.*

The sequence of events in the development of scurvy may be pictured thus: As feces accumulate in the cecum and large intestine of the animal and constipation has set in, peristalsis is decreased and the intestinal juices are secreted in less profusion, resulting in a lowering of the digestive power of the intestine and stomach and probably in the production of lesions in the intestines. Then, due to an increased permeability of the vessel walls, bacteria may invade the joints, as shown by Jackson and Moody,[18] toxic products are absorbed in greater amounts from the cecum and large intestine, and in some cases the whole protein molecule passes through the walls of the capillaries of the intestines into the blood stream.

No claim is made that the retention of feces is the primary factor in the production of scurvy, but it is clearly a contributing and probably a very important factor in the development of the syndrome of this disease.

[18] Jackson, L., and Moody, A. M., *J. Infect. Dis.*, 1916, xix, 511.

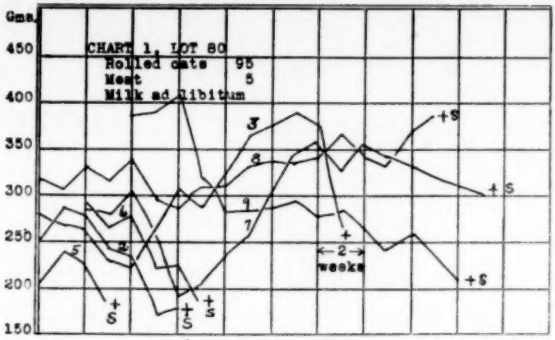

CHART 1. Lot 80 shows the effect of adding 5 per cent of meat to a ration of rolled oats and milk fed *ad libitum*. Animal 5 died at the end of the 3rd week and showed lesions of scurvy. All the other animals developed scurvy during the 4th and 5th weeks. In spite of this, Animals 3, 7, 8, and 9 lived for a considerable time. Animals 7 and 8 were killed at the end of the 17th week. The meat did not delay the onset of the disease but it did prolong the lives of a number of the animals for a long time.

S+ on the charts denotes that the animals had scurvy at the time of death; + signifies death without scurvy.

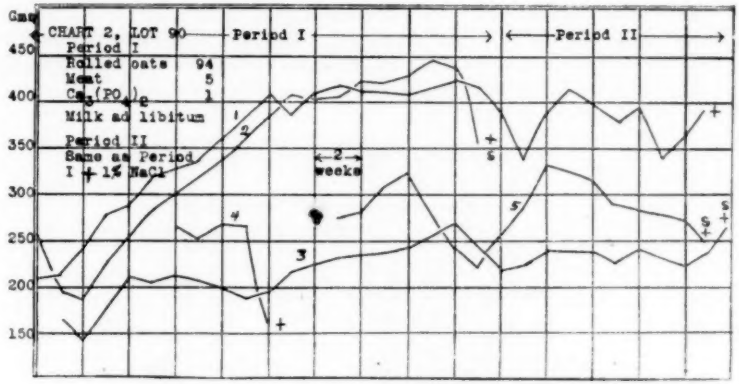

CHART 2. Lot 90. The animals in this lot received in addition to the ration of rolled oats, meat, and milk fed *ad libitum* (Ration 80), 1 per cent of $Ca_3(PO_4)_2$ during the first period. Animal 4 died at the end of the 4th week, but it did not show any lesions of scurvy. No. 5 developed scurvy during the 5th week. The other three animals remained in excellent condition for 18 weeks when they began to lose weight and showed evidences of having scurvy. No. 1, however, although it lost in weight did not develop scurvy. No. 2 died at the end of the 19th week. In Period II, 1 per cent of NaCl was added to the ration. The animals all improved in appearance, became more active, and gained in weight for a time, but the symptoms of scurvy did not disappear. 4 weeks after the ingestion of NaCl into the ration they again lost in weight and Animal 5 died 5 weeks later. Nos. 3 and 1 were killed at the end of the 30th week. No. 3 showed lesions of scurvy while No. 1 did not.

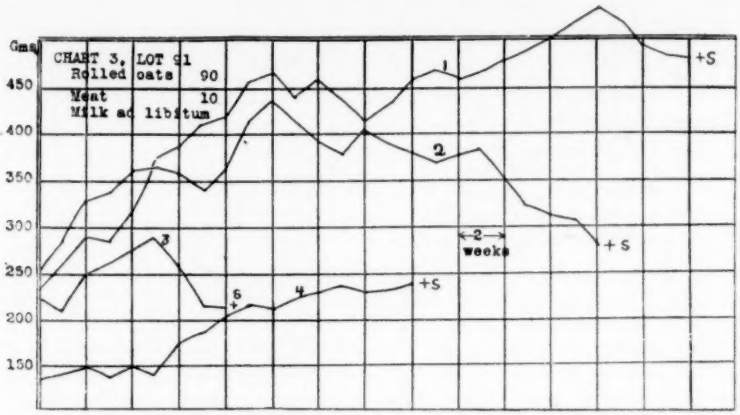

CHART 3. Lot 91 illustrates the behavior of guinea pigs receiving a ration of rolled oats and 10 per cent of meat and milk fed *ad libitum*. Animal 3 developed scurvy during the 5th week and died the 8th week. No. 4 died the 16th week, and No. 2 the 24th week. Animal 1 was killed the 28th week and showed lesions of scurvy. The record of these animals is considerably better than that of the animals that received 5 per cent of meat (see Chart 1, Lot 80), and shows that insufficient protein is ingested in the rolled oats and milk ration.

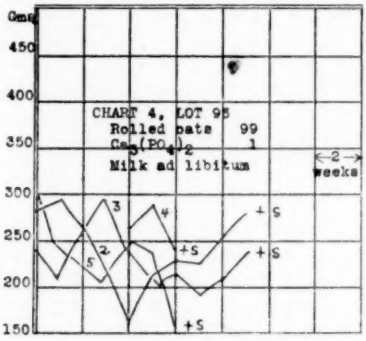

CHART 4. Lot 96 shows the results obtained by feeding $Ca_3(PO_4)_2$ without meat in the rolled oats and milk ration. The $Ca_3(PO_4)_2$ alone was unable to protect the animals against scurvy or to prolong their lives for any appreciable time; but when meat was added to this ration it was very efficient in protecting the animals against scurvy and in prolonging their lives (see Chart 2, Lot 90). Animal 4 died of scurvy at the end of the 2nd week. The other animals all developed scurvy during the 4th and 5th weeks, and No. 5 died at the end of the 6th week. Animals 2 and 3 increased in weight after the 6th week, but were killed at the end of the 9th week.

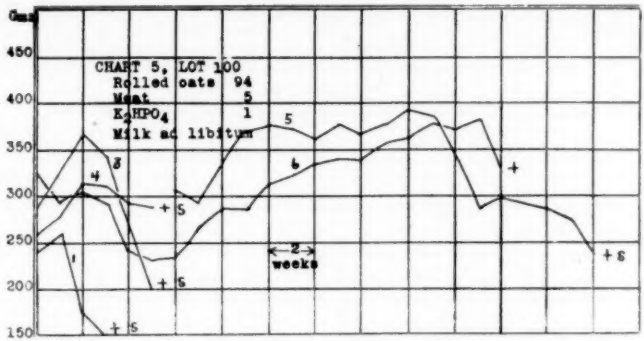

CHART 5. Lot 100 shows the effect of the addition of K_2HPO_4 to the rolled oats, meat, and milk ration. These guinea pigs did little better than did guinea pigs that received only rolled oats, meat, and milk. Animals 1, 3, and 4 developed scurvy during the 3rd week; No. 1 died at the end of the 3rd week, and Nos. 3 and 4 at the end of the 5th week. No. 5 developed scurvy during the 10th week and died the 18th week; No. 6 never developed scurvy. It died at the end of the 19th week, but showed no lesions of scurvy.

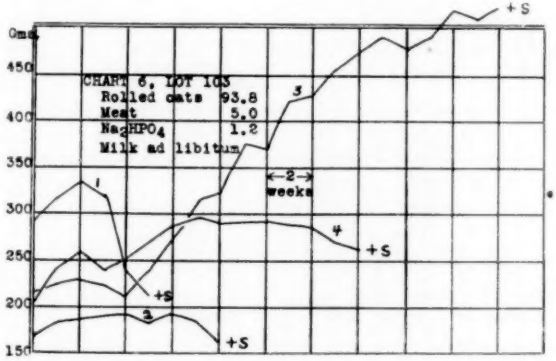

CHART 6. Lot 103. The animals in this group were fed the same ration as those in Lot 100 but in place of K_2HPO_4 they were given Na_2HPO_4 in the ration. These animals did no better than did those that received K_2HPO_4 in this ration (see Chart 5). All the animals except No. 3 developed scurvy during the 4th week. No. 1 died at the end of the 5th week, No. 2 during the 8th week, and No. 4 at the end of the 14th week. Animal 3 grew rapidly and appeared to be normal, but when it was killed it showed a few slight lesions of scurvy.

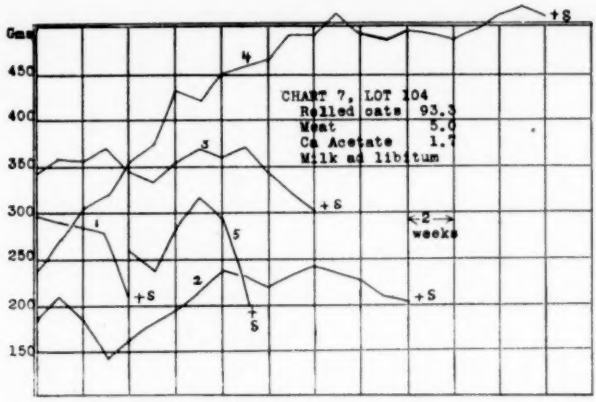

CHART 7. Lot 104 illustrates the behavior of guinea pigs fed the rolled oats, meat 5, and milk ration plus calcium acetate. The amount of calcium in this ration is the same as that fed as $Ca_3(PO_4)_2$ (see Chart 2, Lot 90). All the animals in this group developed scurvy during the 4th week, and No. 1 died at the end of that week; No. 2 died at the end of the 16th week; No. 3 at the end of the 12th week, and No. 5 at the end of the 5th week. Animal 4 grew rapidly for 12 weeks and then maintained its weight, and at no time did it show evidences of having scurvy; but when it was killed at the end of the 20th week it showed a few slight lesions of scurvy. Although the calcium acetate did improve the rolled oats, meat, and milk ration it did not prove as effective as was $Ca_3(PO_4)_2$ (see Chart 2, Lot 90).

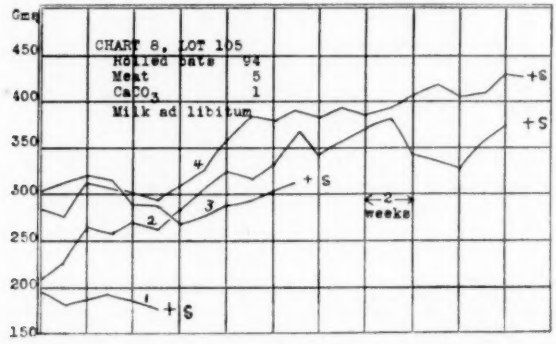

CHART 8. Lot 105 shows that calcium carbonate was no more efficient than calcium acetate in the nutrition of the guinea pig. All the animals developed scurvy during the 5th week and No. 1 died at the end of that week. No. 3 died at the end of the 11th week; No. 2 was killed at the end of the 20th week at which time it had severe scurvy; No. 4 was killed at the end of the 21st week and showed only slight lesions of scurvy. This animal had apparently recovered from the disease to a marked degree.

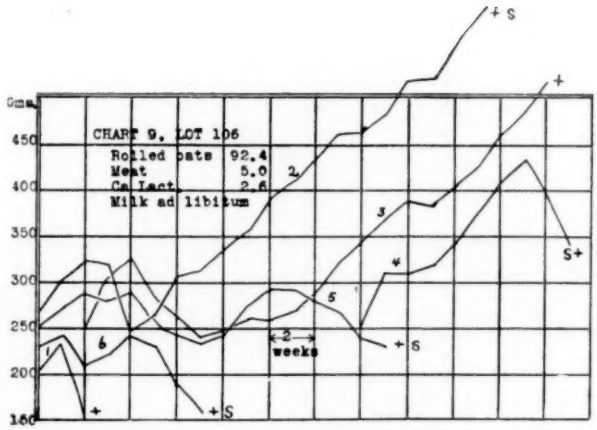

CHART 9. Lot 106 received calcium lactate in place of the carbonate. The curves show that the animals did somewhat better than did those that received calcium acetate (see Chart 7, Lot 104) or those that received calcium carbonate (see Chart 8, Lot 105). Animal 1 died after the 2nd week, but failed to show lesions of scurvy. Animal 2 showed symptoms of scurvy during the 4th week, but it gained in weight rapidly until it was killed at the end of the 20th week and showed severe lesions of scurvy. No. 3 never developed scurvy and grew rapidly. It showed no lesions of the disease when it was killed at the end of the 20th week. No. 4 developed scurvy during the 7th week and died the 9th week. Animals 5 and 6 developed scurvy during the 7th week and No. 6 died at the end of this week, while No. 5 lived until the 16th week.

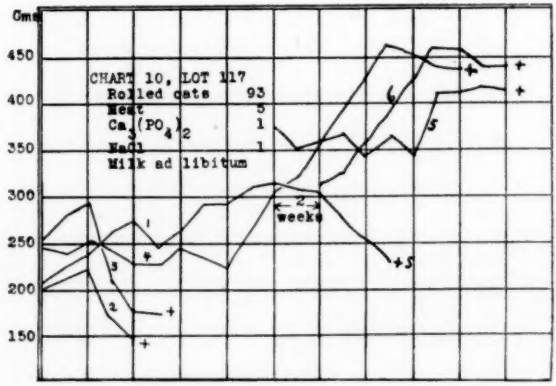

CHART 10. Lot 117. This chart is the record of a group of guinea pigs kept on Ration 80 plus $Ca_3(PO_4)_2$ and NaCl. During the first 4 weeks all the animals except Nos. 4, 5, and 6 developed scurvy. Animal 2 died at the end of the 4th week and No. 3 at the end of the 5th week. Neither of these animals showed lesions of scurvy. Animal 1 died at the end of the 15th week. It was questionable whether or not this animal had scurvy. Animal 4 was discarded after the 18th week, and although it lost weight the last 4 weeks, due apparently to the hot weather, it did not show symptoms of scurvy. Animals 5 and 6 were started on the ration 9 weeks later than the other animals. Animal 5 did not begin to gain in weight until the 7th week after which it gained in weight until the 11th week. The animal, however, developed scurvy during the 9th week. No. 6 grew rapidly until the 5th week when it slowly declined in weight. It was discarded the 8th week and was in good condition at that time.

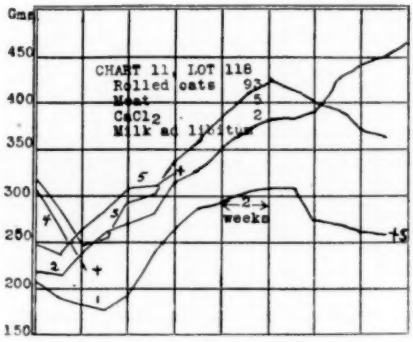

CHART 11. Lot 118 shows the effect of the ingestion of $CaCl_2$ into Ration 80. Animal 4 died at the end of the 3rd week, but showed no signs of scurvy. No. 5 died during the 7th week. The intestine and stomach were greatly distended with gas, but the animal showed no lesions of scurvy. Animals 1 and 5 grew rapidly in weight until the 10th week after which time they slowly declined. They were discarded the 15th week and both showed symptoms of scurvy. Animal 2 grew rapidly throughout the feeding trial and when discarded, the 16th week, it was in good condition. The animals in this group did considerably better than did those that received $Ca_3(PO_4)_2$ and NaCl (see Chart 10).

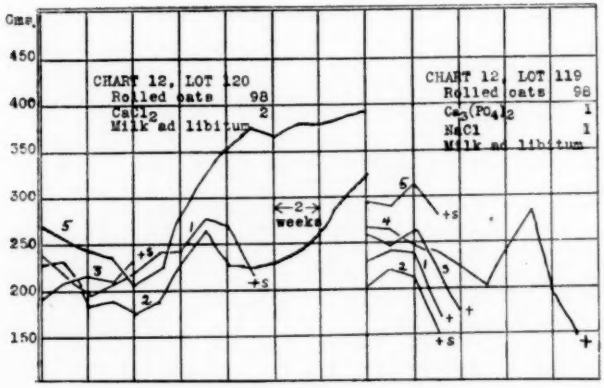

CHART 12. Lot 120 illustrates the behavior of guinea pigs fed upon a diet of rolled oats, $CaCl_2$, and milk fed *ad libitum*. Animal 3 died at the end of the 4th week and showed lesions of scurvy. Animal 1 grew a little but died at the end of the 9th week and showed decided lesions of scurvy. Animal 2 grew at a slow rate and showed symptoms of scurvy the 9th week. Animals 2 and 5 were discarded the 15th week; No. 5 grew until the 9th week after which it maintained its weight, but at no time did it show evidences of having scurvy. These animals did considerably better than those that received $Ca_3(PO_4)_2$ and NaCl (see Lot 119, this chart).

Lot 119 was fed rolled oats, $Ca_3(PO_4)_2$, NaCl, and milk fed *ad libitum*. The animals all lost in weight after the 2nd week, but only one, No. 2, showed symptoms of scurvy. No. 1 died the 4th week, but showed no lesions of scurvy. No. 2 died the same week and did show lesions of scurvy. No. 3 died at the beginning of the 5th week, but showed no lesions of scurvy, while No. 5 died the 4th week and did show lesions of scurvy.

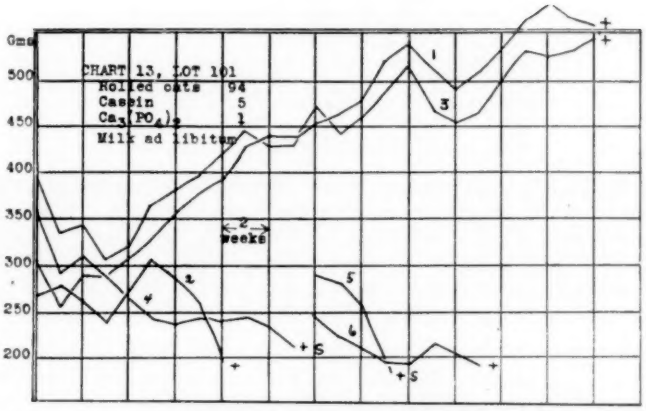

CHART 13. Lot 101 shows the effect of adding casein to a ration of rolled oats, $Ca_3(PO_4)_2$, and milk fed *ad libitum*. These guinea pigs did not do so well as those that received meat in place of casein (see Chart 2, Lot 90), but the casein did supplement the proteins of the ration (compare Chart 4). Animal 5 developed scurvy during the 3rd week and died at the end of that week. No. 4 developed the disease the 9th week and died the 11th week. None of the other animals showed evidence of having scurvy. Animals 1 and 3 were killed at the end of the 24th week and showed no lesions of scurvy.

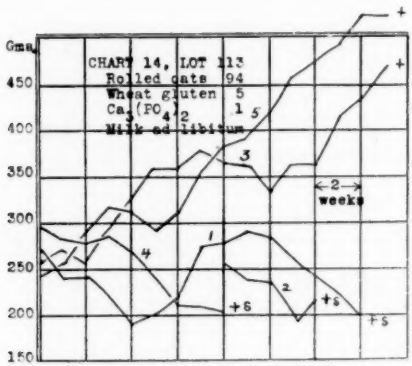

CHART 14. Lot 113 received wheat gluten in place of casein. They did not do so well as did the casein animals (see Chart 13). Three of the animals, Nos. 1, 2, and 4, developed scurvy, while Nos. 3 and 5 showed no evidence of the disease. They were killed at the end of the 15th week and apparently were normal in all respects.

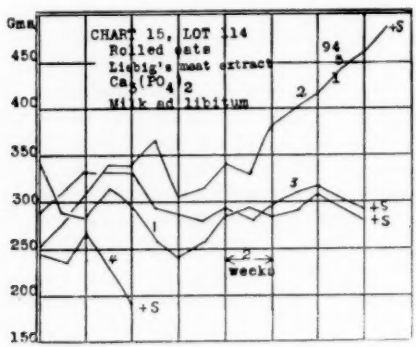

CHART 15. Lot 114 shows the effect of adding Liebig's extract to the rolled oats, $Ca_3(PO_4)_2$, and milk ration. These animals did very poorly and all developed scurvy during the 4th and 5th weeks. No. 2 was killed the 15th week and showed severe lesions of scurvy.

A METHOD FOR THE IDENTIFICATION OF CERTAIN URAMINO-ACIDS IN THE PRESENCE OF AMINO-ACIDS AND OF UREA.

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(From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco.)

(Received for publication, September 25, 1918.)

INTRODUCTION.

The facility with which uramino-acids or their anhydrides, hydantoins, may be formed from amino-acid and urea *in vitro* is shown by the work of Baumann and Hoppe-Seyler (1), Lippich (2), as well as by the critical studies of Salkowski (3), Dakin (4), Weiland (5), and most recently Abel, Rowntree, and Turner (6). Where uramino-acids or hydantoins have been found in biological fluids, urea has been present (7), amino-acids were not excluded, and in some work amino-acids were added with a view to further the synthesis *in vivo* of uramino-acids (8). Due to the fact that certain uramino-acids are compounds difficultly soluble in water and are extracted by ether (9) as well as by acetic-ether (4), they have been obtained as crystalline compounds in procedures for the isolation of other substances such as amino-acids (8, 9, 6), from biological fluids, and have occasionally served as evidence for the presence of amino-acids when these acids could not themselves be isolated.

As the question of the existence of preformed uramino-acids in body fluids has been left open (5, 6), it seemed advisable to undertake to modify the procedure hitherto used for the isolation of uramino-acids by taking advantage of the action of urease (10) for the decomposition of urea and thus secure its elimination from the material to be investigated. It is possible, also, to follow more closely traces of uramino-acids by an application of the Van Slyke gasometric method for the determination of amino nitrogen (11). The uramino-acids studied give off amino nitro-

gen in the course of an hour as contrasted with amino-acids which give it off quantitatively in 4 minutes, with the exception of lysine which takes longer. The anhydrides of the uramino-acids, as was to be expected, give off no amino nitrogen. Therefore by forming anhydrides from uramino-acids, amino nitrogen suspected to be lysine for example, may be identified as having its source from uramino-acid or from amino-acid according as to whether it disappears or remains after the procedure of anhydride formation.

The chief evidence for the presence of preformed uramino-acid is an experiment carried out by Dakin (12) where ureidophenylpropionic acid crystallized from cat's urine without analytical procedure. In this experiment doses of 6 to 8 gm. of phenylalanine were administered intravenously to cats during $1\frac{1}{2}$ hour periods and the urine was collected during the ensuing 11 hours. The urine on standing contained at the edge of the vessel crystals which were identified as ureidophenylpropionic acid. Larger quantities of the uramino-acid were obtained by acidifying the urine with phosphoric acid, extracting in a continuous extractor, distilling the acetic-ether with steam, clarifying with animal charcoal, concentrating the aqueous residue, and allowing to crystallize.

Abel, Rowntree, and Turner (6) in referring to isobutylhydantoin, isolated by them from a dialysate from circulating dog's blood, say: "The isolation of this substance does not necessarily prove its original presence in the blood of the dog, inasmuch as it may have been formed during the ester distillation by a condensation of urea with another substance." The possibility that this other substance may be leucine is pointed out. The question "whether this and perhaps other hydantoin derivatives occur in the animal body" (6) made it desirable to make the following experiments.

A study of α-ureido-β-phenylpropionic acid and of α-ureido-isobutylacetic acid has been carried out. In applying the procedures here described to the question of preformed α-ureido-β-phenylpropionic acid, it was thought advisable to conform as nearly as possible to the conditions under which Dakin isolated 1.5 gm. of this uramino-acid. The same choice of animal, of dosage of phenylalanine, of time of injection of amino-acid, and

of collection of urine was made, and Dakin's method of extraction used.

Method.

The fresh material is treated with Jack bean urease in such proportion as to decompose the urea in 15 minutes (10), at 37°C., or when possible the collections are made into enzyme solutions at this temperature. After testing a portion of the material to make certain all urea is decomposed, the whole amount is neutralized with phosphoric acid and an excess added. The acid fluid is then extracted in a liquid extraction apparatus for 6 or more hours with four to six volumes of acetic-ether. The acetic-ether is then distilled with steam, the aqueous residue clarified with charcoal, and concentrated on the water bath to small volume. Crystals of uramino-acid, if present, are filtered off, dried, and identified by means of the melting point and of amino nitrogen determinations before and after treatment with boiling hydrochloric acid. If no crystals separate and traces of uramino-acids are to be excluded, the concentration may be continued until a small amount of syrup results which is then taken up in 1 to 2 cc. of water, normal sodium hydroxide added drop by drop until alkaline, and filtered if necessary. The material is then boiled for 1 minute to drive off traces of ammonia and made up to volume in a 10 cc. flask. Determinations on the amino nitrogen content may then be made. 2 cc. samples are taken and the determination is carried out as for amino-acid. Second samples of material are shaken 1 minute and then allowed to stand 1 hour in the reaction chamber, and shaken a second time for 3 minutes. If in the second procedure more amino nitrogen is found than in the first a 5 cc. portion of the material is acidified with ten drops of concentrated hydrochloric acid and boiled under a reflux condenser for an hour. The material is made alkaline and again made up to volume and determinations on the amino nitrogen are run. The difference between the amino nitrogen figure before boiling with hydrochloric acid and that after boiling may be assumed to be amino nitrogen from uramino-acid.

EXPERIMENTAL.

A cat weighing 2.35 kilos was given by stomach tube 4.7 gm. of urethane dissolved in warm saline solution. The left femoral

vein was cannulated and connected to a burette containing 300 cc. of normal salt solution in which 6.0 gm. of *dl*-phenylalanine were dissolved. The bladder was exposed, the urethra tied close to the bladder and the latter punctured, the urine withdrawn, and a glass bladder cannula inserted and tied in place. The bladder was replaced in the peritoneal cavity, and the incision closed by clamps. The cannula was so bent that the urine dropped into a 50 cc. beaker which stood in a bath of water at 37–45°C.

The phenylalanine and salt solution were slowly and continuously run into the vein for 1½ hours.

5 cc. of Jack bean extract (10) were placed in the beaker to receive the urine as excreted. This extract was constantly agitated, and the temperature kept between 37–40°C. When 5 cc. of urine had been collected, a fresh specimen of extract was taken for the next collection.

The alkalinity of a 2 cc. portion of the Jack bean extract and urine was determined, and after a 15 minute interval with the material at 37°C., a second 2 cc. portion was titrated, and the completion of the decomposition of urea was verified. Portions of 60 cc. of urea-free material were neutralized with phosphoric acid and 2 to 3 cc. of acid were added in excess and introduced into a continuous extraction apparatus. Approximately 370 cc. of ethyl acetate were in circulation in the extraction apparatus. Extraction was carried on for 6 hours on each specimen, the acetic-ether in the distilling flask (75 cc.) was then removed, and distilled with steam, while the next specimen of acidified material was extracted with the addition of 75 cc. of fresh acetic-ether.

The slightly colored aqueous residues left on distillation with steam were clarified with animal charcoal, filtered, evaporated to 1 to 2 cc. on the water bath, and allowed to stand for 24 hours. No crystallization of uramino-acids took place.

The drops of brown syrup-like material were then examined under the microscope for crystals. Only a trace of amorphous substance was present.[1] The material was taken up in 2 cc. of

[1] Two preliminary experiments, carried out in the same manner as the experiment here reported, gave similar residues with no crystallization of uramino-acid, but the exclusion of uncrystallized uramino-acid in the syrup by the application of the amino nitrogen determination was not done.

normal sodium hydroxide, 5 cc. of water were added, the material was boiled to drive off traces of ammonia present, and then made up to 10 cc. in a measuring flask. Portions of 2 cc. each were analyzed for amino nitrogen.

A bare trace of amino nitrogen was obtained in each residue in the 4 minute determination which was not increased by an hour's reaction with nitrous acid. A control extraction of 50 cc. of normal cat's urine and 50 cc. of Jack bean extract gave a small correction which covered the trace of amino nitrogen found.

Amino nitrogen determinations upon ureidophenylpropionic acid upon its anhydride, benzylhydantoin, upon ureidoisobutylacetic acid and its anhydride, isobutylhydantoin, gave the following figures:

Amount.	Substance.	NH_2-N			
		Time of reaction.			Theory for 50 per cent N.
		4 min.	30 min.	1 hr.	
mg.		*mg.*	*mg.*	*mg.*	*mg.*
16.0	Ureidophenylpropionic acid.	0.26	1.03	1.34	1.07
20.2	Benzylhydantoin.			0	0
10.8	Ureidoisobutylacetic acid.	0.20	0.87	0.94	0.84
23.3	Isobutylhydantoin.			0	0

These uramino-acids were made from amino-acids yielding the theoretical amounts of amino nitrogen. A nitrogen determination on each would indicate their purity; however, slightly more than 50 per cent of the nitrogen of these compounds was usually obtained after 1 hour's interaction with nitrous acid. After 1 hour no more nitrogen was obtained.

Amount.	Substance.	Total nitrogen.	
		Found.	Theory.
mg.		*mg.*	*mg.*
89.4	Ureidophenylpropionic acid.	14.46	14.38
160.4	Ureidoisobutylacetic acid.	22.13	21.82

The specificity of the action of urease has been emphasized by Marshall and figures given to indicate no action upon α-ureido-

β-phenylpropionic acid (10). The following determination shows no action upon α-ureidoisobutylacetic acid. 0.05 gm. of α-ureidoisobutylacetic acid was dissolved in a few drops of normal sodium hydroxide and made up to a volume of 10 cc. with water. 10 cc. of Jack bean extract were added. 4 minute determinations of amino nitrogen were made at once and after 15 hours.

Sample.	Uramino-acid.	Time.	NH_2-N
cc.	*mg.*	*hrs.*	*mg.*
2	5	0	0.33
2	5	15	0.33

Recovery of Added α-Ureido-β-Phenylpropionic Acid.

To 10 cc. of cat's urine and 10 cc. of Jack bean extract which had stood at room temperature 12 hours and was urea-free, were added 0.2 gm. of α-ureido-β-phenylpropionic acid and 1 cc. phosphoric acid, and an extraction experiment was carried out as above.

Crystals separated in the aqueous residue on concentration and after standing 12 hours were filtered from 4 cc. of mother liquor. 0.15 gm. of slightly colored crystals, having the same melting point as the uramino-acid added (185° uncorrected), were recovered. Mixing the synthetic and the product recovered from the urine gave no depression in the melting point. On recrystallizing a part of the product the melting point was not raised. The material was extracted for a further 6 hours, and the acetic-ether in the distilling flask again treated as before. No crystals were obtained from the aqueous residue.

I. Mother liquor from crystalline uramino-acid recovered.
NH_2-N determined in 30 minutes reaction with HNO_2... 1.5 mg.
Uramino-acid equivalent to this N..... 22.0 "
Calculated on a basis of NH_2-N. 50 per cent of total N of compound.

II. Residue from second extraction of original material.
NH_2-N..... 1.0 mg.
Uramino-acid equivalent..... 14.0 "
Total uramino-acid recovered..... 0.186 gm.

0.095 gm. uramino-acid dissolved in normal sodium hydroxide and made up to 10 cc. with water. 2 cc. specimen analyzed.

NH_2-N after ½ hr..... 1.3 mg.

0.095 gm. uramino-acid recovered from extraction experiment dissolved in normal sodium hydroxide and made up to 10 cc. 2 cc. specimen analyzed.

NH_2-N after ½ hr..... 1.3 mg.

Calculated amount of nitrogen for one-half that of uramino-acid..... 1.3 "

Injection of α-Ureidoisobutylacetic Acid.

A cat weighing 2.4 kilos received 5 gm. of urethane by stomach tube and 5 gm. of α-ureidoisobutylacetic acid[2] intravenously during the course of 1½ hours. The urine was collected as described in the experiment on phenylalanine injection. The collections were made over a 10 hour period and the material was examined for uramino-acid as in the previous experiment. A total of 350 cc. of material was obtained and extracted in portions. A considerable quantity of white crystalline substance separated during extraction and crystallized from the aqueous residues before concentration.

A yield of 1.87 gm. of substance was obtained on concentration of the combined aqueous residues.

The material melted at 208° (uncorrected) and gave off amino nitrogen before treating with hydrochloric acid. Isobutylhydantoin made from the uramino-acid melted at 212° (uncorrected) and gave off no amino nitrogen.

		NH_2-N		
		Reaction time.	Found.	Theory for 50 per cent N.
mg.		*hr.*	*mg.*	*mg.*
19.0	Uramino-acid.	½	1.57	1.53
4.6	Hydantoin.	½	0	0

The mother liquor from the crystallization of uramino-acid was made alkaline with normal sodium hydroxide boiled to drive off ammonia and made up to 100 cc. Amino nitrogen determinations indicated the presence of an additional amount of uramino-acid of approximately 0.1 gm.

[2] Made from active leucine.

CONCLUSION.

1. A method has been devised by which one of the disturbing factors in the study of uramino-acids in relation to their biological significance has been minimized, that is the interaction of amino-acid and urea, and Marshall's urease method for the destruction of urea by urease has been applied to a procedure where speed of decomposition with minimum analytical procedure was of especial importance.

2. The Van Slyke method for determining amino nitrogen has been applied to a new series of substances as a means for their identification and study. When associated with the fact that the anhydrides formed by the action of hydrochloric acid on these substances are not decomposed by nitrous acid to give amino nitrogen, the amino nitrogen of uramino-acids may be differentiated from that of amino-acids.

3. Small amounts of several uramino-acids when added to urine have been recovered.

4. The presence of preformed uramino-acid has not as yet been demonstrated in biological fluids following the injection of amino-acids.

5. Uramino-acids have been administered intravenously and recovered from the urine of the cat.

The writer wishes to thank Miss Marion Sweeney for her assistance in the experimental work.

BIBLIOGRAPHY.

1. Baumann, E., and Hoppe-Seyler, F., *Ber. chem. Ges.*, 1874, vii, 37.
2. Lippich, F., *Ber. chem. Ges.*, 1906, xxxix, 2953.
3. Salkowski, E., *Z. physiol. Chem.*, 1880, iv, 107.
4. Dakin, H. D., *J. Biol. Chem.*, 1910–11, viii, 25; *Am. Chem. J.*, 1910, xliv, 55.
5. Weiland, W., *Biochem. Z.*, 1912, xxxviii, 385.
6. Abel, J. J., Rowntree, L. G., and Turner, B. B., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 619.
7. Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.
8. Blendermann, H., *Z. physiol. Chem.*, 1882, vi, 234.
9. Embden, G., and Schmitz, E., *Biochem. Z.*, 1912, xxxviii, 393.
10. Mateer, J. G., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1916, xxv, 297.
11. Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.
12. Dakin, *J. Biol. Chem.*, 1909, vi, 235.

ON A SOURCE OF ERROR IN THE USE OF PICRIC ACID IN COLORIMETRIC ESTIMATIONS IN BIOLOGICAL FLUIDS.

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(Received for publication, September 25, 1918.)

Critical studies of picric acid as used for the quantitative colorimetric determination of creatine and creatinine in blood (1) have been made by McCrudden (2), Hunter and Campbell (3), Folin and Doisy (4), Wilson and Plass (5), Greenwald and McGuire (6), and Denis (7) and suggestions made for modifying the procedure.

In carrying out blood sugar determinations by the Benedict modifications (8) of the Benedict-Lewis method for blood sugar determinations, the following observations were made upon picric acid.

A sample of Baker's chemically pure picric acid was purified by the method suggested by Folin (4). The material was divided into two portions, one put away in a dark closet in a brown bottle, dry (Picric Acid I), the other in the same closet in a colorless bottle with a little moisture (Picric Acid II). When these picric acids were next examined some 10 to 12 months later, the sample contained in the dark bottle was used for sugar determinations. A picric-picrate solution was made up from the second portion in the colorless glass bottle. This solution failed to precipitate protein in the blood samples in the usual way. Apparently less coagulum was formed and the filtrate was more highly colored. A new sample of Baker's chemically pure moist picric acid, Picric Acid III, was made into a picric-picrate solution as before and compared with the two former solutions in blood sugar determinations. Each picric-picrate solution was used also to prepare a glucose standard and compared with a permanent standard of picramic acid.

TABLE I.

Colorimetric Readings on Dog's Blood.

	Blood specimen.	Glucose solution.	Permanent standard.
Picric Acid I {Same original sample.}	10.5	15	15
" " II {Same original sample.}	4.0	15	15
" " III	10.5	15	15

Picric Acids I and III melted at 122° and Picric Acid II at 120°.

TABLE II.

Folin's Color Test for the Purity of Picric Acid.

	Readings after 15 min.		Readings after 24 hrs.	
	Without alkali.	With alkali.	Without alkali.	With alkali.
	mm.	*mm.*	*mm.*	*mm.*
Picric Acid II	10	8.0	10	8.0
" " III	10	9.0	10	9.0

The figures would indicate that Picric Acid II is slightly inferior to No. III, but neither acid is to be discarded from this test. For a pure picric acid Folin gives 20: 14.

Experiment to Test the Acidity of Picric Acids II and III.

Samples of both Picric Acids II and III were titrated with 0.1 N sodium hydroxide, using phenolphthalein as an indicator.

Saturated picric acid solution.	Gm. per 50 cc.	Cc. 0.1 N NaOH necessary to neutralize.
II	0.60	23.5
III	0.60	26.5

The protein of the blood sample was precipitated according to Wilson and Plass (5), and blood sugar determinations were run according to the method of Benedict (8) upon the protein-free neutral material.

Colorimeter Readings.

Picric-picrate solution.	Blood specimen.*	Permanent standard.
	mm.	*mm.*
I	14.5	15
II	14.9	15
III	14.4	15

* 2 cc. of filtrate equivalent of 2 cc. of blood.

CONCLUSION.

A chromogenic substance other than sugar is present in the blood which certain picric acid fails to precipitate. Solid picric acid after purification may undergo a change in its precipitating value for chromogenic substances in the blood. The formation of a deeper color on addition of alkali to picric acid solutions as tested by Folin does not account for the discrepancy of certain picric acids in blood sugar determinations. The precipitating value of picric acid must be determined before reliance may be placed upon color production in quantitative procedures for blood sugar.

BIBLIOGRAPHY.

1. Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.
2. McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxvi, 527.
3. Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1916–1917, xxviii, 335.
4. Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–1917, xxviii, 349.
5. Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.
6. Greenwald, I., and McGuire, G., *J. Biol. Chem.*, 1918, xxxiv, 103.
7. Denis, W., *J. Biol. Chem.*, 1918, xxxv, 513.
8. Benedict, S. R., *J. Biol. Chem.*, 1918, xxxiv, 203.

A METHOD FOR THE ESTIMATION OF POTASSIUM IN BLOOD.

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This paper deals with an adaptation of the cobaltic nitrite method to the determination of potassium in small quantities of organic material.

The cobaltic nitrite method, as developed recently (1–5), includes three steps: (1) precipitation of the potassium as potassium sodium cobaltic nitrite, $K_2NaCo(NO_2)_6.H_2O$; (2) oxidation of this precipitate in acid solution at boiling temperature with an excess of potassium permanganate; (3) titration of the excess of permanganate with oxalic acid.

In attempting to apply this method, it was at once evident that the small quantities of potassium involved (0.2 to 1 mg.) would necessitate the use of rather dilute $KMnO_4$ solution. It was found that such dilute solutions, when boiled with acid, undergo appreciable decomposition. In order to eliminate this source of error, which is variable, use was made of an early and apparently abandoned step in the original method (6).

When the potassium sodium cobaltic nitrite is heated with dilute NaOH, all the nitrite groups are changed to sodium and potassium nitrite, and the cobalt is precipitated as insoluble $Co(OH)_3$ which may be filtered off:

$$K_2NaCo(NO_2)_6 + 3\ NaOH = 2\ KNO_2 + 4\ NaNO_2 + Co(OH)_3$$

The nitrites may be estimated by titration with dilute potassium permanganate. The nitrite solution after cooling is acidified with H_2SO_4 until about normal acidity is reached, and 0.02 N $KMnO_4$ run in until a faint pink color persists for about $\frac{1}{2}$ minute. The titration flask is then warmed on an electric

plate, and permanganate is slowly added until the color persists ½ minute at about 70°C. In a series of analyses of pure $NaNO_2$, in quantities varying from 0.5 to 5 mg. in 25 cc. of solution, the ratios of $KMnO_4$ used to $NaNO_2$ taken did not vary from each other more than 0.3 per cent. Furthermore, $NaNO_2$ in such amounts as 0.5 to 5 mg. may be boiled with 1 per cent NaOH for 5 minutes without appreciable loss of reducing power for $KMnO_4$.

In the presence of normal acidity, at room temperature, the reducing power for $KMnO_4$ remains apparently unchanged for 2 or 3 minutes, although the odor of the oxides of nitrogen is apparent. Of course at elevated temperature, the loss of reducing power is very rapid in acid solution.

These facts furnish the basis for the estimation of small quantities of potassium.

Estimation of Potassium in Pure Solutions.

The reagents are:

1. Sodium Cobaltic Nitrite Reagent.—This (7) is prepared from two stock solutions which keep well several months.

A. Cobalt nitrate	50 gm.
Glacial acetic acid	25 cc.
Distilled water to	100 "
B. Sodium nitrite	100 gm.
Distilled water to	200 cc.

6 parts A and 10 parts B are mixed. A rapid current of air is passed through the solution for several hours to remove the fumes of oxides of nitrogen. Then the dark brown fluid is kept on ice 2 days. Invariably some yellow precipitate forms, owing to traces of potassium and ammonium in the reagents. The filtered reagent keeps well for several weeks, if in a dark bottle in an ice box.

2. Potassium Permanganate.—This is prepared (8) by diluting 0.1 N $KMnO_4$ to about 0.02 N and boiling under a funnel reflux condenser for 2 to 3 hours. After 1 to 2 days, the solution is decanted from the manganese oxides which have separated, and preserved in a cool dark place. Such solutions keep well several weeks.

3. Standard Potassium Solutions.

A. Standard, 1 cc. = 10 mg. potassium.

Pure KCl	9.546 gm.
HCl (concentrated)	1 cc.
Water to	500 "

B. Standard, 2 cc. = 1 mg.

Standard A	25 cc.
HCl (concentrated)	1 "
Water to	500 "

The potassium chloride is recrystallized several times, then fused. The hydrochloric acid is added to prevent growth of moulds.

The solution for analysis should contain 0.2 to 2.5 mg. of potassium. It is measured into a 50 cc. beaker and evaporated nearly but not quite to dryness, on a water bath. Two or three drops of glacial acetic acid are added, and 1 cc. of the sodium cobaltic nitrite reagent. Evaporation is continued until the mixture becomes syrupy. It is then cooled, and 4 to 5 cc. of water are added. One should not carry the evaporation too far, as insoluble brown compounds are formed, which are liable to be associated with too high results. The solution is cooled and 4 or 5 cc. of water are added.

The precipitate of potassium sodium cobaltic nitrite is filtered off on a Gooch crucible with a fairly thick asbestos mat. Filter paper must not be used, because during the subsequent boiling with 1 per cent NaOH, considerable reducing substance is formed from it. Just before the mat is sucked dry, a suspension of $BaSO_4$ is poured onto it. This not only fills up the larger pores, but serves as an excellent test of the mat. Before the water has all passed through the mat, the contents of the beaker containing the potassium sodium cobaltic nitrite are poured on. The beaker is rinsed with 4 to 5 cc. of cold water, and the rinsings are poured onto the nearly dry mat. Such washing, with 2 to 3 cc. of water, is repeated six times. The mat when almost dry is transferred to the beaker, and all adhering particles are washed from the crucible to the beaker with 9 to 10 cc. of water. 1 cc. of 10 per cent NaOH is added, and the contents of the beaker are heated to boiling, then cooled. The dark brown mixture is made up to exactly 25 cc. and centrifuged. 20 cc. of the clear fluid are pipetted off into a 150 cc. Erlenmeyer flask, 5 cc. of

1:4 H_2SO_4 added, and titration with 0.02 N $KMnO_4$ as described above is at once carried out. Blanks, run frequently, are usually small (0.05 to 0.10 cc.). The potassium value of the permanganate is determined by analysis of the standard solution B. This value should be determined frequently, but does not vary greatly from day to day. Calculations are based on the values thus obtained rather than on a theoretical factor.

As may be seen from Table I, the analysis of pure KCl solutions is satisfactory.

TABLE I.

Analysis of Pure KCl Solutions.

Potassium taken, *mg.*	0.25	0.50	0.75	1.00
0.02 N $KMnO_4$ required, *cc.*	1.53	3.08	4.59	6.13
0.02 N $KMnO_4$ per mg. K, *cc.*	6.12	6.15	6.13	6.13

Estimation of Potassium in Blood.

In applying this method to blood, it was thought that the salts present might interfere, although Green (9) had shown that this was not the case in applying the cobaltic nitrite method to urine. I have found that Ca, Mg, Fe, and phosphates, in quantities as great as would be met with in blood analysis, do not interfere with the accuracy of the determination when added to pure KCl solutions. Organic material is removed by a "wet" ashing process.

The blood is ashed in a 200 × 25 mm. Pyrex glass tube with a mixture of nitric and sulfuric acids. For 2 cc. of plasma, or 1 cc. of blood, 5 cc. of a mixture of sulfuric acid, 1 part, nitric acid 20 parts, are sufficient. The tube arranged with a fume absorber as for a Folin micro-Kjeldahl determination is heated by a microburner, adjusted so that boiling is slow, for about ½ hour. Foaming and bumping can be largely prevented by the use of a short piece of platinum wire sealed through the bottom of the tube, serving as a heat-conductor and boiling focus. After ½ hour the excess of HNO_3 is rapidly evaporated. The remaining drop of sulfuric acid darkens considerably. At this stage, the flame is turned off, and the small amount of HNO_3 condensed on the walls of the tube allowed to run back. If this is not sufficient to clear

up the H_2SO_4, two to three drops of HNO_3 are added and boiling is resumed until the acid is colorless, when the tube is cooled.

The oxidation mixture is washed into a 50 cc. beaker, made alkaline to phenolsulfonephthalein with 10 per cent NaOH, and evaporated to dryness on the water bath. A few drops of glacial acetic acid are added, until the mixture is acid; then 1 cc. cobaltic nitrite reagent is added, and evaporation continued until crystals

TABLE II.

Recovery of Potassium Added to Serum.

	mg.
1. Serum alone	0.61
Added K(as KCl)	1.00
Total	1.61
Found	1.62
2. Serum alone	0.70
Added K(as KCl)	1.00
Total	1.70
Found	1.70

TABLE III.

Potassium Determination in Blood, Expressed as Mg. per 100 Cc.

Diagnosis.	Plasma.	Whole blood.
Circumcision	52.8	200
Nephritis (?)	67	222
Diphtheria	65	
Pott's Disease		178
Nephritis		165
Cystitis; bladder stone	117*	234
Intestinal obstruction	89.5	265
Pyelitis	70.5	290
Alimentary intoxication	71.8	143

* Slight hemolysis had occurred.

of Na_2SO_4 appear. The mixture is then cooled. Dehydration by means of the Na_2SO_4 results in very complete precipitation of the $KNa_2Co(NO_2)_6$. From this point the determination is carried out as for pure solutions already described.

That potassium added to blood may be recovered is evident from Table II. A few results obtained with plasma and whole blood are given in Table III.

In conclusion, it is to be emphasized that the reagents must be tested for the presence of potassium. One sample of C. P. NaOH purified by alcohol contained nearly 0.05 per cent potassium. The sodium citrate used as an anticoagulant must also be tested for the presence of potassium.

BIBLIOGRAPHY.

1. Bowser, L. T., *J. Am. Chem. Soc.*, 1911, xxxiii, 1752.
2. Bowser, *J. Ind. and Eng. Chem.*, 1910, i, 791.
3. Adie, R. H., and Wood, T. B., *J. Chem. Soc.*, 1900, lxxvii, 1076.
4. MacDougall, F. H., *J. Am. Chem. Soc.*, 1912, xxxiv, 1684.
5. Shedd, O. M., *J. Ind. and Eng. Chem.*, 1911, ii, 379.
6. Sutton, F., A systematic handbook of volumetric analysis, Philadelphia, 10th edition, 1911.
7. Hamburger, H. J., *Biochem. Z.*, 1915, lxxi, 415.
8. Halverson, T. O., and Bergeim, O., *J. Ind. and Eng. Chem.*, 1918, xix, 119.
9. Green, H. H., *Biochem. J.*, 1912, vi, 69.

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